Microalgal adaptation to changes in carbon dioxide

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Table of Contents

Abstract

It is generally accepted that global levels of CO_2 will roughly double over the next century. Because of their large population sizes and fast generation times, microalgae may adapt to global change through novel mutations fixed by natural selection, such that future populations may be genetically different from contemporary ones. The prediction that microalgae may respond evolutionarily to rising CO_2 was tested using populations of *Chlamydomonas reinhardtii* grown for 1000 generations at increasing CO_2. Laboratory populations grown at high CO_2 did not show a direct response to selection at elevated CO_2, instead evolving a range of non-adaptive syndromes. In addition, populations selected at elevated CO_2 often grew poorly at ambient CO_2. The same evolutionary responses were seen in natural populations isolated from CO_2 springs. CO_2 uptake was measured in a subset of the laboratory selection lines, which were found to have cells that either leaked CO_2, had lost the ability to induce high-affinity CO_2 uptake, or both. These phenotypes were tentatively attributed to the accumulation of conditionally neutral mutations in genes involved in the carbon concentrating mechanism (CCM). The high-CO_2-selected phenotypes were found to be reversible in terms of fitness when populations were backselected in air, though wild-type regulation of the CCM was not regained. It has been suggested that phytoplankton adaptation to changes in CO_2 levels is constrained by selective history. This was tested by culturing genetically distinct populations of *Chlamydomonas* at decreasing levels of CO_2. In this case, divergence between lines was attributable to chance rather than selective history.

Résumé

Au cours du prochain siècle, il est convenu que le taux atmosphérique global de gaz carbonique (CO_2) doublera. Les algues microscopiques, ou microalgue, caractérisées par de larges populations ainsi qu'un court laps générationnel, pourront s'adapter à ce changement global par la fixation de mutations aléatoires sous l'effet de la sélection naturelle. Ainsi, les futures populations de microalgues pourront être génétiquement différentes des populations présentes. Une telle réponse évolutive à l'augmentation du CO_2 chez les microalgues a été testée sur des populations expérimentales de *Chlamydomonas reinhardtii* cultivées dans un environnement où le CO_2 a été augmenté sur 1000 générations. Les populations expérimentales cultivées à haut taux de CO_2 n'ont pas démontré de réponse directe à cette pression sélective, mais plutôt des syndromes non adaptifs. De plus, ces populations démontrent généralement une faible croissance à un taux de CO_2 ambiant. Par ailleurs, des réponses évolutives similaires ont été observées chez des populations naturelles de microalgues isolées à partir de sources naturelles à haut taux de CO_2. Certaines cellules d'un sous-ensemble des populations expérimentales mesurées pour la capture du CO_2 laissent fuir ce dernier, alors que d'autres ont perdu l'habileté d'induire une réaction de capture du CO_2 à haute affinité, ou encore que certaines cellules présentent les deux syndromes. Ces phénotypes sont provisoirement attribués à l'effet de l'accumulation de mutations neutres et conditionnelles à l'intérieur de gènes impliqués dans le mécanisme de concentration du carbone de ces microalgues. Bien que le mécanisme de concentration du carbone n'ait pas été restauré, les phénotypes associés aux population sélectionnées à haut taux de CO_2 ont démontré que la valeur

sélective associée à ces populations est réversible lorsqu'elles sont cultivées dans leur environnement initial (air ambiant). Finalement, il a été suggéré que l'adaptation du phytoplancton au taux de CO_2 est contrainte par son historique de sélection. Cette hypothèse a été testée en cultivant des populations génétiquement distinctes de *Chlamydomonas* à des taux décroissant de CO_2. Dans cette expérience, les divergences entre les populations expérimentales ont été attribuées au hasard plutôt qu'à l'historique de sélection.

Acknowledgements

I am unsure of how I ended up working with Graham Bell, since I remember intending to be a biochemist, so I shall have to make do with saying that I am grateful to whatever happy accident of undergraduate life landed me in his office near the end of my degree. I could thank Graham for all that I have learned from him since my arrival at McGill. Which I do. He is one of the best teachers that I have ever had. But I should also like to thank him for his calm vote of confidence over the not-always-smooth course of my Ph.D.

Dieter Sültemeyer provided valuable help with the physiological work in this thesis, including, but not limited to: a crash course in carbon uptake kinetics and physiology, sharing the secrets of the mass spec and associated arcane devices, and his infectious enthusiasm for all things green. I also appreciate the help of the members of my supervisory committee at McGill: Neil Price, Tom Bureau and Cathrine Potvin, who were especially important in encouraging me to make my work more accessible to those in the related disciplines of ecology and oceanography.

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Thesis Format and Contributions of Authors

A PhD candidate at McGill University may submit a thesis based on a series of manuscripts on which the student is an author or coauthor. Having chosen this format, I am obliged to explicitly state who contributed to each paper and to what extent.

The introduction immediately following this section presents the motivation and rationale for this study, and provides the required integration of the chapters that follow. Chapter 1, "Timing is everything: phytoplankton responses to CO_2 enrichment over different temporal scales" is a literature review which will be submitted to *Trends in Ecology and Evolution* in the coming weeks as a paper by S. Collins. The original idea for the paper was mine; I prepared the first draft of the manuscript, then Graham and I jointly revised the manuscript.

Chapter 2, "Phenotypic consequences of 1,000 generations of selection at elevated CO_2 in a green alga," was published in *Nature* in 2004 (431:566-569) as a paper by S. Collins and G. Bell. This is the selection experiment on which the rest of the thesis is based. The original idea for the selection experiment was Graham's, and had been previously attempted in collaboration with Catherine Potvin. Graham suggested the project to me at the end of my undergraduate degree. Graham and I discussed how to improve the experimental design, and planned the selection experiment together. I set up and ran the experiments with technical assistance from K. Tallon and various work-study students, analyzed the results in consultation with Graham, and wrote the rough draft of the

manuscript. Mark Romer and Claire Cooney at the McGill Phytotron provided technical support with the growth chambers used for the experiments and assays throughout the thesis.

Chapter 3, "Changes in carbon uptake in populations of *Chlamydomonas reinhardtii* selected at high CO_2," has been submitted for publication in *Proceedings of the Royal Society B; Biological Sciences* as a paper by S. Collins, D. Sültemeyer, and G. Bell. This chapter uses the same selection lines that were used in Chapter 2. I executed and analyzed the assays with valuable technical help and discussion from Dieter. I wrote the manuscript after discussion with Graham and Dieter; Graham and I then edited the manuscript.

Chapter 4, "Rewinding the tape: selection of algae adapted to high CO_2 at current and Pleistocene levels of CO_2," has been submitted to *Evolution* as a manuscript by S. Collins, D. Sültemeyer and G. Bell. This experiment was designed to expand on work done in chapter 2. I designed, coordinated and analyzed the experiment and wrote the manuscript with valuable advice and discussion from Graham. A pilot experiment was done by Z. Machanda as an undergraduate project. Substantial help (making media, washing flasks) from work-study students was needed in order to carry out the selection experiment, though I performed all of the assays. Dieter provided equipment and discussion on the design and interpretation of the physiology assays.

Chapter 5, "Evolution of natural populations of algae selected at elevated CO_2," has been submitted to *Ecology Letters* as a manuscript by S. Collins and G. Bell. I collected or requested the soil samples, performed the experiments and analysis and wrote the manuscript, which Graham then edited.

To my knowledge these all constitute original contributions to knowledge, except of course the review in Chapter 1, which largely summarizes and attempts to integrate the work of others, though it also contains some original ideas and suggestions.

Introduction

It is generally accepted that atmospheric CO_2 levels will roughly double over the next century. About half of global carbon fixation occurs in oceans, which suggests that phytoplankton have the potential to fix a portion of anthropogenic CO_2. Because this could help control global CO_2 levels, there has been great interest in studying phytoplankton responses to elevated CO_2. Phytoplankton responses to elevated CO_2 are usually studied: 1) in order to characterize inorganic carbon uptake in contemporary phytoplankton, 2) to investigate the effect of CO_2 concentration on community composition, or 3) in order to make predictions about the properties of future populations. To date, experimental studies have focused exclusively on either physiological responses or the outcome of short-term competition experiments, the results of which are then scaled up to produce predictions about future populations. However, an explicit test of how elevated CO_2 affects microalgae over hundreds or thousands of generation requires it's own set of experiments. Since mutation and natural selection may act over the time scale that global change occurs on, the work in this thesis uses experimental evolution to study long-term responses to changes in CO_2 in microalgae by describing the end phenotypes and the evolutionary processes that may result in such phenotypes. Chapter 1 is a literature review that focuses on responses to elevated CO_2 over different temporal scales, Chapters 2 and 3 are concerned mainly with describing high-CO_2-selected phenotypes, Chapter 4 discusses the roles of historical constraints and chance in adaptation to changes in CO_2, and Chapter 5 examines adaptation to elevated CO_2 in natural populations.

The vast majority of the data available on phytoplankton responses to changing CO_2 levels come from short-term studies, making one of the challenges of this work to integrate ideas, data, and methods from fields that traditionally study different time scales: physiology, ecology, and evolution. Physiology studies changes that occur within generations, ecological studies in this field tend to span several days (tens of generations), whereas evolutionary studies can span hundreds or thousands of generations. Each time scale of study has it's own set of assumptions and methodologies that are used in order to make predictions about future phytoplankton populations. These are reviewed in Chapter 1.

Throughout this work, I have focused on experimental rather than comparative approaches partly because the rate of CO_2 increase is much more rapid than it has previously been, so that the Anthropocene (current era) has no true analogue in the geological past. This implies that the rate of environmental change itself affects adaptive outcomes. While this seemed (perhaps naively) to make intuitive sense to me, I was unable to find any explicit treatment of how the rate of environmental change may affect adaptation by the fixation of novel mutations. Based on work characterizing the size of fixed beneficial mutations, I propose a verbal explanation for why slow and abrupt environmental change may produce different end phenotypes in the second half of Chapter 1.

Since phytoplankton have large population sizes and short generation times, they may respond to elevated CO_2 by changing genetically as a result of novel mutations fixed by natural selection. Thus, an evolutionary response to rising CO_2 has the potential to introduce an error of unknown sign and magnitude into predictions regarding future phytoplankton populations. This idea is

introduced and tested in Chapter 2, which describes the evolutionary outcome of long-term growth under rising CO_2 in an algal model system. I used a standard laboratory selection experiment in order to evaluate if rising CO_2 can drive adaptive evolution in microalgae. For the selection experiment, I used *Chlamydomonas reinhardtii*, a unicellular green alga that is a well-characterized model system for carbon uptake and photosynthesis, as well as for experimental evolution studies. We found a number of non-adaptive evolutionary responses that differed in sign, magnitude, or both from the short-term environmental (acclimation) response. For example, the environmental response to elevated CO_2 is to increase cell size, but the evolutionary response is to decrease it. Surprisingly, there was no adaptive response to elevated CO_2, though some of the High selection lines showed a rather spectacular and obvious negative correlated response where they grew poorly or (occasionally) failed to grow at all in air. The same evolutionary response was found in natural populations isolated from CO_2 springs, which are described in Chapter 5.

Because there was an increase in variance but no increase in mean fitness in the High selection lines, we suggested that increasing CO_2 relaxed stabilizing selection and allowed the accumulation of conditionally neutral mutations in the experimental lines. Since the High selection lines could fix CO_2 when it was abundant, we hypothesized that the carbon fixation machinery itself was still functional and that cells had somehow lost the ability to take up CO_2 at lower external concentrations. If this were the case, it would be theoretically possible for the lines selected at high CO_2 to rely more on diffusion in a high CO_2 environment without experiencing any decrease in intracellular CO_2 levels. The hypothesis that CO_2 uptake had been degraded in the High selection lines is tested in Chapter 3, where inorganic carbon uptake is

measured separately from CO_2 fixation using a mass spectrometer. Using this method, I determined that the High selection lines had an impaired Carbon Concentrating Mechanism (CCM, an inducible system by which most microalgae actively take up inorganic carbon) and were composed of cells that were either leaky or incapable of inducing high-affinity CO_2 uptake, or both. The presence of a functional, inducible CCM is the cornerstone of many predictions of phytoplankton responses to elevated CO_2, and our work suggests that this fundamental character can change over hundreds or thousands of generations of exposure to elevated CO_2. This chapter provides both evolutionary and physiological explanations of High selection phenotypes.

To this point the thesis focused on non-adaptive phenotypes resulting from growth at high CO_2. While it is relatively straightforward, results in hand, to explain why no adaptation to elevated CO_2 occurred, there was initially no reason to suppose this would be the case. In fact, upon seeing the (negative) results, my first response was not to attempt to explain them but to repeat the fitness assays more carefully. Chapter 4 follows this up by examining the role of constraints, specifically selective history, on adaptation to changes in CO_2. History is thought to constrain adaptation by limiting the number of beneficial changes accessible from a given starting point. Selective history is thought to be important to adaptation to elevated CO_2 in plants, and it has been suggested that specific adaptation to limiting CO_2 precludes adaptation to current or future levels. The evolved selection lines from Chapter 2 had different selective histories or starting points (High and Ambient). These lines were selected for growth at decreasing CO_2, which eventually reached levels last experienced during glaciation in the Pleistocene. The results from this experiment do not support the hypothesis that selective history constrains adaptation. All lines were able to adapt to low CO_2 regardless of selective history, and did so to roughly the

same extent. In addition, divergence between selection lines was attributable mainly to chance rather than to ancestor.

A special case of how selective history affects adaptation is reverse evolution, or reversion to an ancestral state. Evolution should be irreversible, given that the probability of retracing a specific series of mutational events is small, as is the chance of serendipitously ending up at the ancestral genotype by some other route. (This is a simplistic argument that deserves further thought. However, I have focused on the if and how High selection phenotypes are reversible, rather than a thorough exploration of the underlying theory. I have begun working on the theory, but it is outside both the scope and timeline of this thesis.) In cases of reverse evolution, lines that have adapted to a novel environment that are then selected in the ancestral environment (air) tend to return to the ancestral fitness, but not to the same underlying genotype or phenotype. Reverse evolution is particularly relevant to microalgal adaptation to CO_2 because CO_2 varies cyclically over many time scales (geological, or just a single bloom). Thus, reverse selection of some sort is likely to be a general feature of microalgal adaptation over most time scales, and may help to explain current biology. The High selection lines from Chapter 2 were used to test the hypothesis that while changes in fitness would be reversible, underlying phenotypic changes would not. The High selection lines were grown at CO_2 levels that were gradually decreased until they reached ambient levels. The results of this experiment provide support for the above explanation, in that the lines that had undergone reverse selection returned to the same fitness as lines that had remained at ambient CO_2 for the same amount of time, but did not regain regulation of CO_2 uptake that had been lost during growth at elevated CO_2. Instead, the lines became specifically adapted to ambient CO_2, which also occurred in the control lines. This in good agreement with

reverse selection experiments in other systems where fitness is recovered, though the ancestral phenotype is not.

The entire premise of this thesis is that laboratory experiments using a model organism can be used to gain useful information about processes that occur in the real world. This is hardly an unusual assumption or research approach. Experimental evolution in well-studied model organisms is often used in order to better understand general processes such as adaptation, sexual selection, or the maintenance of diversity. However, other experiments are intended to describe and explain phenotypes that are ecologically, commercially, or medically important. Phytoplankton responses to elevated CO_2 fall into the latter category: they are interesting primarily because they are likely to have some ecological relevance, rather than as a way to explain some other more general evolutionary process. As such, it is important to evaluate whether the outcome of these laboratory selection experiments produce responses to elevated CO_2 that can also occur in natural populations. Chapter 5 describes the growth of natural populations of microalgae isolated from CO_2 springs and compares them to the High selection lines obtained in the laboratory selection experiment. Like the laboratory selection lines, the natural populations do not show any adaptation to elevated CO_2, but have lower fitness at ambient CO_2. At least in terms of fitness, *Chlamydomonas* appears to be a good model system for studying evolutionary responses to elevated CO_2 in microalgae.

Linking section 1

Most publications on the topic of phytoplankton (or land plant) responses to elevated CO_2 begin with something closely resembling the following sentence: It is well-accepted that global atmospheric CO_2 will roughly double over the next century. This sentence contains two statements. The first is an estimate of the magnitude of environmental change that is expected. The second is an estimate of the rate that this change will occur at. However, it is generally the reaction to the magnitude, rather than the rate, of CO_2 enrichment that is the focus of study. Many of the publications then go on, after presenting their specific data, to make some sort of prediction about the properties of future populations.

The following chapter, "Timing is everything: phytoplankton responses to CO_2 enrichment over different temporal scales" reviews published experimental studies on phytoplankton responses to elevated CO_2, with the goal of critically examining the basis for predictions that are made about the properties of future populations of phytoplankton. Though there are many studies of phytoplankton responses to elevated CO_2, the majority of experimental data available comes from short-term studies, which are then scaled up in order to predict long-term changes in the properties of phytoplankton populations. The first part of this chapter reviews the information available at increasing time scales, and compares the resulting predictions. Most of the experiments reviewed (all save one) study the response to a step change in CO_2, and so inform us about how phytoplankton respond to a given magnitude of environmental change, but tell us little about how the rate of environmental change is likely to affect adaptive outcomes. The second part of chapter 1 presents a verbal explanation for one way that rates of environmental change could affect adaptive outcomes.

Chapter 1: Timing is everything: phytoplankton responses to CO_2 enrichment over different temporal scales.

This chapter will be submitted to *Trends in Evolution and Ecology* as an Opinion paper by S. Collins. The section **Physiological responses to elevated CO_2** has been expanded for the purposes of this thesis, which requires an extensive literature review. The version of this section that will be submitted for publication can be found in the appendix at the end of this chapter.

Abstract

Phytoplankton responses to elevated CO_2 is an active research question in several different fields of biology, including plant physiology, ecology, oceanography, population genetics, and evolution. Since phytoplankton responses to CO_2 enrichment are of significant practical concern, experimental data is often extrapolated to make predictions about the phenotype or composition of future populations. We briefly review experiments carried out over increasing temporal scales, how they have been used in the formulations of future predictions, and the connections that have been made between them. Common to most experiments is the assumption that sudden and gradual changes in CO_2 produce similar phenotypes. We suggest that the rate of environmental change in itself may be important to adaptive outcomes over long time scales and propose a verbal explanation for this based on differences in the sequential fixation of beneficial mutations.

Introduction

Since the industrial revolution, man has had an unprecedented impact on global biogeochemical cycles, partly through the addition of carbon dioxide to the atmosphere. This has led to an increase in global atmospheric CO_2 of approximately 90ppm since the beginning of the industrial revolution, with the largest increase occurring during the past century (see for example Watson et al., 2001). Anthropogenic influence on the carbon cycle also impacts climate, other nutrient cycles, and photosynthesis in terrestrial and aquatic systems. The current era has been aptly named the Anthropocene and since it has no true analogue in the geological past, it is difficult to use historical data in order to understand biotic responses to current global change. In addition, biotic responses to rising CO_2 have the potential to occur on several different time scales,

ranging from physiological to evolutionary, each of which has it's own theoretical framework, standard assumptions and methods.

About half the photosynthetic carbon fixation on the planet occurs in oceans (Beardall and Raven, 2004; Falkowski, 1994). Phytoplankton have the potential to absorb a portion of anthropogenic CO_2 as fixed organic carbon and trap it in deep ocean sediments (Raven and Falkowski, 1999; Sarmiento and Toggweiler, 1984), though debate exists over the size of such a sink. The biological carbon sink has generated interest in terms of its possible role in slowing global CO_2 enrichment, and has given rise to large-scale experiments designed to increase phytoplankton blooms and subsequent sinking by adding iron dust to a section of the Southern ocean (Boyd et al., 2000; Buesseler et al., 2004; Coale et al., 2004). Experiments like this confirm a growing interest in the idea that phytoplankton may have the potential to help manage global CO_2 enrichment. Because CO_2 enrichment occurs on large temporal and spatial scales, understanding how phytoplankton will respond to CO_2 enrichment requires biologists to take these problems of scale into account, especially when considering large-scale environmental manipulations such as iron enrichment.

Problems of scale are inherent to biology: in order to understand the individual processes underlying adaptation, experiments are done in relatively short-lived, simple systems. The conclusions from those experiments are then used to understand processes that occur in long-lived, complex environments. Laboratory experiments are not meant to be miniature, accelerated versions of oceans, but are instead meant to simplify processes enough to explain them. When a simplified system is understood well enough that biologists can predict its behavior, that

understanding is then applied to natural systems. In order to understand the processes that govern phytoplankton reactions to CO_2 enrichment, biologists must scale up from short-term studies, yet almost no empirical tests of how these processes scale up over hundreds or thousands of generations exist.

Here, we discuss recent experimental studies that describe the responses of phytoplankton to CO_2 enrichment over physiological, population genetic and evolutionary time scales (see Box 1), and the predictions about phytoplankton responses to increasing CO_2 that have been made based on these studies. When little or no empirical data from microalgae is available, data from higher land plants is used providing it is reasonable to suppose that responses may be similar despite obvious differences in biology. As such, large parts of the literature on higher plant responses to elevated CO_2 are ignored here. We discuss how processes occurring at one time scale are likely to affect those at longer time scales, and the impact this could have on the predictions made at each level. In comparing short and long-term responses to elevated CO_2, we observe that short-term responses are unlikely to scale up in a predictable manner, at least in some cases. We also suggest that the rate of environmental change itself can affect the outcome of adaptation, and propose an explanation for how this may occur. How marine processes sensitive to CO_2 enrichment may scale up in space has recently reviewed by Riebesell (2004). Here, we focus on how phytoplankton responses may scale up in time.

Physiological responses to elevated CO_2

The most widely-used definition of phytoplankton responses to changing CO_2 is a physiological one. Physiological responses describe immediate changes in the rates and affinities of carbon

uptake and fixation within generations, or changes in population growth rates over short periods of time. It is important to note that a large proportion of physiology studies are designed to explain the current responses of phytoplankton to short-term fluctuations in CO_2, for example over the course of a bloom, or to characterize how inorganic carbon is taken up, and are not meant to be predictive over longer time scales. Experiments have been carried out in both laboratory and natural populations and have been extensively reviewed (see for example Raven, 1991; Beardall et al., 1998; Riebesell, 2004).

Most microalgae and cyanobacteria have a carbon concentrating mechanism (CCM) that allows them to rapidly acclimate to changes in external CO_2 levels A difference in photosynthetic characteristics between low- and high-CO_2 cells was first described in 1968 (Graham and Whittingham, 1968), using *Chlorella* cells acclimated to different levels of CO_2. Following that, work in *Chlamydomonas* found that cells grown in air showed higher carbonic anhydrase activity than cells grown at higher concentrations of CO_2 (1%) (Nelson et al., 1969). The correlation between the changes in photosynthetic characters and carbonic anhydrase activity was then demonstrated in several different species of microalgae and cyanobacteria (Graham and Reed, 1971; Graham et al., 1971; Döhler, 1974; Ingle and Colman, 1975; Findenegg, 1976). This strongly suggested that some sort of inducible system for controlling the uptake of inorganic carbon existed in phytoplankton, and that this mechanism existed across a wide range of species. Following this, the ratio of internal/external carbon was shown to be higher than would be possible by diffusion in *Chlamydomonas* and *Anabena* (Badger et al., 1977, 1978), confirming that these microalgae were actually concentrating inorganic carbon inside their cells. Some form

of CCM has been found in most microalgae and all extant cyanobacteria studied to date (Coleman et al., 2002; Miyachi et al., 2003).

Increases in CO_2 lead to decreased affinity for inorganic carbon, sometimes accompanied by a shift from bicarbonate to CO_2 uptake. In short, most phytoplankton respond to carbon limitation by inducing a carbon concentrating mechanism. Conversely, the CCM is inactive at elevated concentrations of inorganic carbon. The CCM enables microalgae to have a high apparent affinity for inorganic carbon, and has the end effect of saturating Rubisco, the first and rate-limiting step in photosynthesis. The CCM has several components, such as energy-dependent carbon transporters, subcellular compartmentalization and a series of carbonic anhydrases (CA) (Badger et al., 1980; Aizawa and Miyachi, 1986; Tsuzuki and Miyachi, 1989; Sültemeyer et al., 1993; Suzuki et al., 1994; Badger and Price, 1994; Spalding, 1998). The number, location, and affinity of CAs vary from species to species. Some species show both extra- and intracellular CA, such as *Chlamydomonas reinhardtii* and *Duniella tertiolecta*; the extracellular CA enables them to take up bicarbonate and dehydrate it for use in photosynthesis (Miyachi and Shiraiwa, 1979; Tsuzuki et al., 1980; Miyachi et al., 1983; Imamura et al., 1983; Tsuzuki, 1983; Aizawa et al., 1986). In contrast, other microalgae have only intracellular CA, and cannot use bicarbonate. These include *Chlorella vulgaris* and *Chlorella miniata.* The location(s) and affinity of carbonic anhydrase vary from species to species (Reed and Graham, 1981; Aizawa and Miyachi, 1986; Fukuzawa et al., 2000; Hewett-Emmet, 2000). While there is still some ambiguity about the function of some carbonic anhydrases, particularly the periplasmic CA in *Chlamydomonas* (Moroney et al., 1985; Williams and Turpin, 1987; Spalding, 1998; Van and Spalding, 1999), it

is agreed that internal (chloroplast) CA is necessary for photosynthesis in eukaryotic microalgae (Funke et al., 1997).

Despite the lack of subcellular compartmentalization in the strict sense, cyanobacteria also posses a CCM that functions much in the same way as a microalgal one. Several reviews have been published on characters of the cyanobacterial CCM (Kaplan et al., 1990; Colman, 1991; Badger and Price, 1992; Ogawa, 1993 and Miyachi et al., 2003). In cyanobacteria, the CCM involves the active transport of bicarbonate under limiting CO_2, and maintains a high bicarbonate concentration in the cytoplasm (Badger et al., 1985; Volokita at al., 1984; Abe et al., 1987). Since bicarbonate does not leak out through the cell membrane, this creates a large pool of inorganic carbon that can be dehydrated to form CO_2 and used for photosynthesis (Kaplan and Reinhold, 1999).

The carbon concentrating mechanism is an inducible system that requires protein synthesis and energy for the active transport of inorganic carbon (Sültemeyer, 1998; Badger et al., 1998; Badger and Spalding, 2000; energetic costs in Raven et al., 2000; Beardall and Giordano, 2002). Both the affinity and inducibility of the carbon concentrating mechanism vary between species (Burkhardt et al., 2001; Colman et al., 2002; Badger et al., 2002; Rost et al, 2003). When CO_2 becomes more abundant, the affinity of the CCM is often reduced, presumably to avoid paying an unnecessary cost of protein synthesis and active uptake. In *Chlamydomonas*, the CCM is active in air, but not at elevated CO_2 (Bozzo and Colman, 2000). Since some microalgae also actively take up bicarbonate, increases in CO_2 concentration can allow microalgae to shift from bicarbonate to CO_2 uptake; this is thought to be because once CO_2 is abundant, its uptake

requires less energy than does bicarbonate uptake (van Hunnik et al., 2002; Tortell et. al. 2002; Burkardt et al., 2001; Rost et al., 2003). Changes in CCM activity at elevated CO_2 is a highly variable but well-studied response in pure cultures.

In addition to changes in carbon uptake, growth at elevated CO_2 influences other processes that may affect fitness or relative fitness in microalgae. Some of these physiological changes have a clear relationship to carbon uptake, and involve the fixation and storage of carbon. The proportion of Rubisco in the pyrenoid increases at limiting CO_2 (Borkhsenious et al., 1998), along with an increase in net photosynthesis rates. There are also substantial changes in starch accumulation pattern (Thyssen et al. 2001). In addition to these changes in carbon fixation and storage, growth at elevated CO_2 has effects on cellular organization that are thought to be related to changes in CCM activity. These include an increase in pyrenoid starch sheath (Kuchitsu et al., 1998; Ramazanov et al., 1994) and changes in mitochondrial distribution, where the mitochondria move from a central to a peripheral position (Geraghty and Spalding, 1996). This is thought to be important since several genes encoding mitochondrial proteins are upregulated at the same time as this relocation (Eriksson et al., 1996; Geraghty and Spalding, 1996; Spalding et al., 2002). It was suggested that the pyrenoid starch sheath was a barrier to CO_2 diffusion (Badger and Price, 1994), but later experiments showed that it was not necessary for normal CCM operation (Villarejo et al., 1996).

In a more general sense, it has been suggested that changes in CO_2 levels may change the use of other nutrients (Raven, 1991) such as light, nitrogen use, and the uptake of trace nutrients.

However, in an experiment using *Emiliana huxleyi* grown for 19 days at three different levels of CO_2, phosphate and nitrate assimilation was similar at all levels of CO_2 (Engel et al., 2005). Other physiological responses may have an indirect effect on fitness. For example, growth of natural populations at elevated CO_2 has also resulted in an increase of transparent exopolymer material (Engel 2002). As well, the ratio of particulate inorganic to organic carbon decreased with increasing CO_2 in laboratory cultures (Riebesell et al., 2000; Zondervan et al., 2001, 2002), which was attributed to a decrease in calcification rates. As is discussed later, it has been suggested that these types of changes will affect the amount of CO_2 being produced by calcification, they could provide a significant negative feedback loop to increasing CO_2 (Engel et al. 2005).

A carbon concentrating mechanism that buffers the carbon fixation machinery from changes in external carbon levels should result in phytoplankton whose growth rates are insensitive to increases in CO_2 (Tortell and Morel, 2002; Cassar et al., 2004). However, phytoplankton are usually responsive to CO_2 enrichment in some way (Clark and Flynn, 2000; Beardall et al., 1998). In some cases, CO_2 enrichment has been shown to increase primary productivity in natural populations of phytoplankton (Hein and Sand-Jensen, 1998). In other cases, there is no effect of CO_2 on primary productivity, but there is an effect on phytoplankton biochemistry or physiology such as changes in calcification, nitrogen use, or the production of extracellular polymers, as detailed above. Though many phytoplankton may show an absence of a short-term growth response to CO_2 enrichment, there is ample evidence that increases in CO_2 correlate with physiological change in phytoplankton, and that many of these physiological changes have the

potential to affect interactions between phytoplankton as well as the physical environment should they be sustained over long periods of time.

In addition to laboratory experiments, physiology experiments measure the effects of CO_2 enrichment on natural communities, either in bottles or in ocean enclosures over the course of a bloom. This allows biologists to better interpret the results of laboratory experiments, which typically use high-density monospecies cultures, as well as to evaluate the effect of changes in CO_2 in combination with other associated abiotic changes, such as carbonate chemistry. It also incorporates feedbacks between biotic and abiotic systems that occur over several days. Mesocosm experiments conducted in outdoor seawater enclosures studying the effects of CO_2 enrichment found that *E.huxleyi* blooms were sensitive to CO_2 increases, and reported increases in instantaneous growth rate, though not in total biomass. Decreases in calcification and preferential loss of carbon over other nutrients were also seen (Engel et al., 2005). In an earlier study using bottle incubations over several days, no increase in growth rate or species composition was seen in response to CO_2 enrichment, though short-term carbon fixation rates differed between CO_2 treatments (Tortell et al, 2000). These experiments are important because they demonstrate that even though natural assemblages of phytoplankton are not thought to be CO_2 limited, CO_2 enrichment still causes changes in characters that may be correlated with fitness, which would allow natural selection to act on these populations.

Physiology-based predictions about future phytoplankton populations are based on changes in relative fitness that may occur as a result of a sustained or predictably changing physiological response. The main predictions that result from physiology studies address questions of if and

how CO_2 enrichment will affect a given species and often speculate about how this in turn will affect larger scale processes such as competition or abiotic feedbacks. In terms of methods and interpretation of data, one representative example of using physiological data to speculate about the properties of future populations and their effect on global CO_2 levels by Tchernov et al. (1998) used microalgae grown in artificial seawater. In this study, CO_2 and bicarbonate fluxes were measured in several different microalgae grown in either artificial or filtered seawater, containing current amounts of dissolved inorganic carbon. Under the conditions of the experiments, sustained CO_2 efflux occurred along with photosynthetic CO_2 fixation. Based on measurements of contemporary microalgae adapted (and acclimated) to current conditions, the authors suggest that future marine microalgae may constitute a source of CO_2 rather than a sink. In this case, the behavior of contemporary populations under contemporary conditions is directly scaled up to make a prediction about future populations under different conditions. A second example of a physiology-based prediction relies on acclimation responses to elevated CO_2. This has been used to suggest that species that are carbon-limited will benefit most from increasing CO_2 levels (Rost et al., 2003). Here, cultures were acclimated to a given level of CO_2 for at least three days, after which photosynthesis and inorganic carbon uptake rates and affinities are measured. (Figure 1) Here, photosynthesis is not saturated at lower CO_2 levels in coccolithophores, so it is predicted that they would experience the largest change in relative fitness under higher CO_2 conditions, since they will be able to increase CO_2 uptake and photosynthesis in response to rising CO_2.

Physiology-based predictions are based on acclimation responses, and have underlying assumptions that the basic physiology of the species being studied will remain unchanged (or

behave predictably) over long periods of time, both in that induction or repression of various processes will be more or less sustained, and that no substantial genetic change affecting the response at the population level will occur over the time scale of interest. As will be discussed, competition and experimental evolution experiments can be used to directly test these assumptions.

CO_2 enrichment and competition

If physiological responses to elevated CO_2 vary between taxa, then future taxa should have a different relative fitness from their contemporary counterparts. If this is the case, increasing CO_2 should change the relative abundance of different types. For example, this could favor taxa that rely more on CO_2 (Burkhardt et al., 2001; Rost et al., 2003), and is supported by the succession of phytoplankton groups over geological time (Tozzi et al., 2004). Competition experiments where two or more species are cultured together for several generations at different levels of CO_2 can be used to directly test this hypothesis. While the studies in the previous section characterize responses within a single generation (or over very few generations) and are usually measured over a few minutes, interactions between taxa, such as competitive exclusion, occur over several generations and allow enough time for changes in the relative abundance of types due to differences in fitness.

The hypothesis that elevated CO_2 could result in changes in the composition of mixed populations was not found to be true over 3 to 6 day incubations of natural populations of

phytoplankton at several levels of CO_2 (Tortell, et al. 1997, 2000). However, over 11 days, and with a different initial assemblage of species, a significant shift in the relative abundances of phytoplankton taxa was seen in response to CO_2 enrichment (Tortell, 2002). The shift in relative abundance was repeatable between microcosms, in agreement with the shift in composition being attributable to systematic differences between taxa. These results suggest that the responses of phytoplankton communities may depend on the initial community composition, as is predicted by physiological data, as well as the total time allowed for the competition. This is not surprising, as the speed with which one type displaces another depends on the difference in mean fitnesses between categories of phytoplankton being scored. As will be discussed shortly, the mean fitness of a population itself may respond to elevated CO_2, such that the rate or outcome of a short-term competition may not reflect the rate or outcome of a longer-term one.

Populations containing genetic variance for fitness should respond to CO_2 enrichment as better-adapted genotypes already present in the base population replace less-adapted types. This within-population sorting complicates between-population competition because the mean fitness of a population in a given environment can change over time. The rate of change depends on both the strength of selection and the genetic variance present in the base population, and increases with increasing values of both (Fisher, 1958). Evaluating how important this process is likely to be in natural populations requires some knowledge about the genetic variance for fitness in these populations. It is possible to measure variance in natural phytoplankton populations, though genetic variance is generally used in order to determine population structure, and is rarely connected to variation in fitness (Iglesias-Rodriguez et al., 2002; Fuller et al., 2005; Evans et al, 2004; Rynearson and Armbrust, 2004; Medlin et al., 2000; vanOppen et al., 1996). Measures of

within-population genetic variance in fitness could be used in order to evaluate which populations are more likely to be able to adapt to CO_2 enrichment by changing their genetic composition. Adaptation can be hindered by migration, though in cases where population structure has been studied, there does not appear to be significant genetic exchange between geographically isolated populations of the same species of phytoplankton (Iglesias-Rodriguez et al., 2002; Rynearson and Armbrust, 2004).

The relative abundance of different taxa has the potential to influence ecosystem functions that are important in global change, such as the ability of the ocean to sequester CO_2. The effect of calcifying phytoplankton, such as coccolithophores, is unique in the carbon cycle. Calcifying phytoplankton create a carbonate counter pump that releases CO_2 into the atmosphere, in opposition to the organic carbon pump, which removes CO_2 from the atmosphere (see figure 2). Despite the transport of carbon to the deep ocean, calcification results in the net release of CO_2 into the atmosphere because the precipitation of calcium carbonate lowers the alkalinity of seawater, leading to water that is over-saturated with CO_2 (Zondervan et al., 2001). The relative strengths of the carbonate pump and the organic carbon pump (called the rain ratio) determines biologically-mediated ocean-air CO_2 exchange. However, decreases in calcification at elevated CO_2 suggest that the CO_2 storage capacity of the surface ocean will increase, not decrease (Riebesell et al., 2000). Levels of calcification could further change if calcifying phytoplankton change in abundance as well as calcification efficiency.

The main assumption in a population genetic approach to phytoplankton responses to elevated CO_2 is that de novo mutation does not contribute to the total genetic variance of a population. This is a reasonable assumption over tens or possibly a few hundred generations, but as will be

discussed in the next section, is probably not reasonable to assume over the time scale that CO_2 enrichment occurs on. It is worth noting that very short-term competition studies also rely on a sustained physiological response, but this can be taken into account by simply allowing the competition to continue for longer. This does not discount the information gained by population studies: that changes in CO_2 levels have the potential to change the rank fitnesses of different taxa which can lead to a rapid change in the composition of mixed populations. This, together with geological data, strongly suggests that shifts in relative abundance are likely to be important as CO_2 levels rise, especially when these shifts are based on large differences in biology between taxa, such as using either calcium carbonate or silicate for external structures. While competition studies can yield valuable information about which taxa are likely to benefit most from rising CO_2, they cannot predict whether any novel change in properties such as growth or CO_2 uptake will occur within taxa.

Evolutionary responses to rising CO_2

At long time scales it is possible that novel phenotypes emerge as a result of mutation, resulting in physiology outside the range of current populations. Exclusion of one species by another is the result of differences in relative fitness that exist in current populations; it requires no shift in genotypes within populations and no mutation. In principle, the outcome of competition should be predictable if the relative fitnesses of the species involved are known. This predictability depends on physiological responses to rising CO_2 remaining fixed or changing in a known way over the time scale of interest. The contribution of mutation to adaptation can be ignored over short time scales, but over the time it will take for CO_2 to double, hundreds or thousands of

generations of phytoplankton will occur. Marine phytoplankton often have population sizes of 10^{12} or larger, and generation times of days (Lande, 1981). If a beneficial mutation rate on the order of 10^{-9} per cell per generation is assumed (Imhof and Schlötterer, 2001), there is ample time for novel mutations to occur and fix in populations. Because evolutionary responses depend on mutation, which is a stochastic process, they introduce uncertainty into predictions of long-term responses to CO_2 enrichment.

Long-term adaptation to increases in CO_2 has been tested experimentally in *Chlamydomonas reinhardtii* over 1000 generations (Collins and Bell, 2004; Collins et al., unpublished). The high CO_2 selected populations evolved two syndromes characterized either by increased photosynthesis without increased growth, or insensitivity of growth and photosynthesis to CO_2 levels (figure 3). The high selected lines studied lost the ability to induce high affinity CO_2 uptake, and on average formed smaller populations than did the control populations. These responses are examples of the evolution of physiology outside the range present in the ancestral population, as well as significant divergence between initially similar replicates. In this case, physiological responses are poor predictors of evolutionary responses, since they can differ in both magnitude and sign from evolutionary responses. This has also been found to be true in natural populations of land plants growing around CO_2 springs, where short-term responses are often attenuated or even reversed after several generations of exposure to high CO_2 (microalgal responses in Collins and Bell, unpublished; responses in multicellular plants reviewed by Urban, 2003). In general, results from experimental evolution and work in CO_2 springs do not support the assumption that physiological responses are likely to remain constant or change predictably over hundreds of generations.

Since species differ in their response to CO_2 enrichment, it has been suggested that species with little plasticity in growth responses to CO_2 will change genetically if CO_2 rises, whereas species that are capable of a plastic response will rely on that instead (Bradshaw and MacNeilly, 1991; Kingsolver 1996). This assumes species that have a plastic response to changes in CO_2 would be under weaker selection pressure (would be closer to a fitness optimum) than species that cannot. Recent experimental work suggests that populations possessing an active carbon concentrating mechanism at current levels of CO_2 that allows them to respond physiologically to changes in CO_2 evolve by accumulating neutral change that erodes phenotypic plasticity, resulting in populations that grow in a narrower range of CO_2 levels than the ancestral populations (Collins and Bell, 2004, Collins et al., unpublished), drawing attention to the possibility that plasticity, like any other trait, may change over evolutionary time. Also, the hypothesis that species showing more plasticity are less likely to adapt genetically implies that the cost of a the plastic response is low, such that sustaining an acclimation response without adaptive genetic change leads to higher fitness than adapting through genetic change. However, the attenuation or reversal of acclimation responses seen after long-term growth at high CO_2 has been reported (Polle et al., 2001; Collins and Bell, 2004), which suggests that the ability to acclimate to elevated CO_2 over one or a few generations may not be a reliable indicator of the strength of selection acting over an extended time.

Experimental evolution and populations isolated from naturally occurring high CO_2 environments can be used to inform predictions about future phytoplankton populations, as well as to test some of the assumptions and hypotheses put forward in physiological and competition

studies. Selection experiments have been used to determine if and how CO_2 can act as a selective agent, and can also be used to generate a range of possible high-CO_2-selected phenotypes. This is the case with the experiments described above. Selection experiments can also be used to predict which physiological processes may scale up in time and which may not by comparing the outcomes of long-term and short-term studies. Practically, long-term selection experiments can be carried out over a time scale that is appropriate for testing many physiology-based hypotheses regarding the properties of future populations, such as the possibility that increasing CO_2 levels will lead to increased resource-use efficiency in phytoplankton (Raven, 1988, 1990, 1991a,b; Beardall et al., 1998). Though many hypotheses have been put forward regarding how phytoplankton are likely to evolve (or fail to evolve) as CO_2 rises, few of these have been tested. Using experimental evolution to do so is straightforward and would result in a more realistic evaluation of phytoplankton responses to elevated CO_2.

The largest limitation of experimental evolution work on microalgal adaptation to high CO_2 is the lack of published studies, leaving no basis for determining how much variation exists in evolutionary responses within or between taxa, as well as how biotic interactions could affect these responses. Work in terrestrial systems studying plant communities near naturally occurring CO_2 springs suggests that long-term responses to CO_2 enrichment vary idiosyncratically between species (Bettarini et al., 1999). In contrast, populations of microalgae isolated from CO_2 springs showed no effect of genus on growth rates or maximum population densities (Collins and Bell, unpublished). Genetic differences have been found between plants occurring at CO_2 enriched and non-enriched sites (Woodward, 1999), as well as systematic reductions in growth rates in microalgal populations at CO_2 enriched sites (Collins and Bell, unpublished). In addition, the

attenuation or reversal of physiological responses has been seen in some land plants growing at CO_2 springs (Balaguer et al., 1996; Bettarini, 1999), which supports the findings of the experimental study described above. CO_2 springs are comparable to long-term mesocosm experiments, and suggest that evolutionary processes contribute to adaptation to CO_2 enrichment even when other processes such as competition and migration occur.

Time scale of environmental change

The rate of environmental change itself may influence adaptive outcomes. Most experiments and subsequent predictions regarding CO_2 enrichment are based on responses to a single step increase in CO_2, whereas over the next centuries CO_2 levels will rise incrementally. This will expose each generation of phytoplankton to a minute, perhaps imperceptible, increase in CO_2 concentration, but it is unclear whether abrupt change in the laboratory is a reasonable representation of the gradual change that occurs in the real world. The time scale of anthropogenically-mediated CO_2 enrichment lies somewhere between two empirical points of reference: short term experiments, and geological data. However, to the best of our knowledge, there are no published experimental tests of the effect of the rate of environmental change on adaptive outcomes where novel genetic change occurs. Population genetic models have examined the effect of the rate of environmental change on sorting and chances of extinction, and have found that the ratio of the mutation rate and the genetic variance in fitness determine the maximum rate of environmental change a population can sustain (Pease et al., 1989; Lynch et al., 1991; Boulding and Hay, 2001). It may be possible to use these models, with mutation

simply incorporated as additional genetic variance, but the dynamics and outcome of the fixation of novel mutations often differ from those of sorting existing variance.

There are several reasons that the rate of environmental change itself could affect adaptive outcomes over longer periods of time. These reasons, along with possible outcomes, are summarized by figure 4. The assumptions made are as follows: First, that adaptation to an unchanging novel environment happens first by the fixation of beneficial mutations of large effect, then of smaller effect, then of even smaller effect etc., with adaptation following a "decreasing returns" scenario. This has been shown to occur in large microbial populations (Gerrish, 2001). Second, the number of possible beneficial mutations decreases with the magnitude of effect of these mutations (Orr, 2002). Third, the ancestral populations are initially well-adapted to their environment, such that any change in the environment causes a decrease in adaptation, either absolutely or through changes in rank fitness. The size of the decrease is positively correlated with the amount of environmental change. Finally, I assume that the environment is perceived by the organism as having more or less continuous rather than threshold values. Although enzymes may be induced or repressed at a threshold value, fitness does not remain constant as the environment changes from one threshold value to the next. An example of this may be that carbon is actively transported when it is present below a given external concentration and passively diffuses when present above that external concentration. However, as external carbon becomes more and more abundant, the fitness advantage associated with active transport may decline continuously, even if the induction of active transport does not.

Starting with a well-adapted population, two possible types of environmental change can occur over a fixed period of time. One is a rapid step change, and the second is a gradual change. The total magnitude of environmental change experienced over a given time is the same in both cases; only the rate differs. The gradual change can be considered to be a series of smaller step changes, with the size of the step determined by the turnover time of the population relative to the rate of environmental change. This is reasonable if the environment changes slowly with respect to the generation time of the organism, such that each generation (or group of generations, as in the case of a phytoplankton bloom) experiences a constant global environment (or the same range of environments), but the average global environment experienced by distant generations will be different. In the case of a single large change, fitness will decrease suddenly, and be regained over time through a series of mutations of decreasing effect. In the case of the gradual change, fitness will repeatedly decrease by small amounts and be regained by mutations of small effect. A second possibility in the case of gradual change is that beneficial mutations of small effect may not fix rapidly enough, as selection pressure will be low, and so the fitness of the population may decrease over several "steps" before a mutation fixes, leading fewer fixed mutations than in the first gradual change scenario. In terms of the size of mutations fixed, the initial mutation following an abrupt change causes a large increase in fitness, but in the case of gradual change (or small environmental change), the initial mutation causes a much smaller increase in fitness, assuming that it fixes at all. This is because the population is assumed to be moving towards the same adaptive peak no matter how quickly the environment is changing; sudden change simply moves the population further away from the adaptive peak (produces a larger drop in fitness) than does a small change.

The reasoning behind the assumptions made about the size of beneficial mutations, as well as the process by which they fix, is outlined by Orr (2002) and is based on Gillespie's mutational landscape model (1983, 1984, 1991). Here, adaptation is characterized by an approximately exponential distribution of effects of fixed mutations, such that the mean phenotypic effect of fixed adaptive mutations falls off as an approximate geometric sequence (Orr 1998, 1999). Prior to a change in the environment, the population is fixed at wild-type, which is the fittest local type, meaning that it was more fit than all of the types that were a single mutational step away. Following a change in the environment, the rank fitness of the wild-type allele decreases by some number *i*, but remains near the top in fitness (see Figure 5). This assumption is reasonable since environments are usually autocorrelated in time, so that it is unlikely that the best adapted type one day will be very poorly adapted the next. In addition, most mutations will give rise to unconditionally deleterious mutations that will decrease fitness regardless of the environment. It is unlikely that the wild-type will be worse than these. Following the drop in rank fitness of the wild-type, natural selection will then move the population from type *i* to some fitter type. In the case presented here, a sudden (large) change in environment results in a larger value of *i* than does a gradual (small) environmental change. The case of gradual environmental change is simply several iterations of a small environmental change, where the new fixed allele (*i*-n), where n represents some type more fit than *i*, is the new wild-type at the beginning of each iteration. It is important to note that the types accessible to the new wild-type were inaccessible to the initial wild-type, as they differ by two mutations. Gillespie (1984) emphasized that, to a good approximation, only sequences a single mutation away were accessible (see also Burch and Chao 2000). This may break down at extremely large population sizes or mutational rates, as in retroviral populations (Cuevas et al. 2002).

Several differences in adaptive outcomes are evident between rapid and gradual environmental change. Sudden changes are more likely to result in the fixation of mutations of large effect than are gradual changes. It follows that if there are more possible mutations of small effect than there are mutations of large effect, the populations experiencing gradual change should diverge genetically more than those that have experienced a step change. With large populations, every possible mutation occurs every generation. However, beneficial mutations have a low probability of fixation, which depends on the selective value of the mutation (Haldane, 1927; derived for competing beneficial mutations in Gerrish and Lenski, 1998). Because of this, though some beneficial mutation will eventually fix in a large population where they are constantly arising, the fate of individual mutations of small effect is heavily influenced by chance. This creates genetic differences between populations early on, where each mutation fixed creates a unique genetic background for the next mutation to fix in. Epistatic effects will then affect the selective value of subsequent mutations, leading to further divergence between populations (Muller 1939; Mani and Clarke 1990; Korona 1996). This will occur in populations experiencing both sudden and gradual change. However, mutations of small effect are more prone to the vagrancies of chance, such that the best of many mutations may not fix at all. In addition, there are more mutations of small effect possible, and a greater total number of mutations (greater total number of iterations of new types) are fixed between the ancestral and final populations in the case of gradual change. Thus, divergence is likely to be greater in the populations experiencing gradual change. Since a small environmental change results in a smaller drop in fitness, it may also mean that phenotypes are accessible only by mutations of large effect are much less likely to occur in populations experiencing gradual change.

In terms of changes in fitness, the populations subjected to sudden changes only adapt to a single environment, while the populations experiencing gradual change adapt to a series of intermediate environments. As described above, adaptation to intermediate environments could constrain adaptation to subsequent environments, whereas the populations experiencing sudden change will be constrained only by adaptation to the initial (ancestral) environment. The number of descendants of a given lineage over time is multiplicative, so the correct measure of the fitness of a population or lineage over time is the geometric mean fitness. In the case of rapid environmental change, the geometric and arithmatic mean fitness of the population are equal, with the lineage that does best in the final environment being the most fit. However, in the case of gradual environmental change the most fit lineage is not necessarily the one that does the best in the final environment, but the one that avoids doing poorly in any one of the intermediate environments. This suggests that a slow environmental change may lead to a less-adapted phenotype than does a rapid change. Practically, experiments using sudden changes in CO_2 may overestimate the degree of adaptation or specialization possible in the end environment. A "bet-hedging" strategy has been proposed as a single processes that links micro- and macroevolution since it occurs on all time scales (Simons, 2002). This makes it useful for examining changes in fitness over timescales where several different processes (physiological responses, competition, natural selection, stochastic events) occur simultaneously. It would, however, require that changes be described in terms of fitness, which is not always possible to calculate based on physiological measurements.

Conclusions

The main practical conclusion that can be reached by comparing responses reported over increasing temporal scales is that short-term processes may not scale up reliably. This is most easily seen in physiological responses that are attenuated or reversed over longer timescales, such as trends in cell size at elevated CO_2. This may also occur in the outcome of competitions between populations because of the possibility for genetic change within populations following sorting or mutation, though this has not been directly tested. Because phytoplankton responses to elevated CO_2 are likely to be ecologically important, the straightforward use of experimental results from short-term studies is unlikely to produce a realistic description of future populations, which strongly suggests that the uncertainty introduced by evolutionary processes should be explicitly taken into account.

Problems of scale introduce a tradeoff between having a clear prediction and a realistic one. Practically, longer processes introduce uncertainty. For short-term processes, uncertainty can be reduced by increasing the number of species studied or the realism of the experiments, for example by using seawater enclosures or natural populations. While this can offer a more complete description of short-term responses, long-term responses are influenced by inherently unpredictable processes such as chance events (either man-made or natural) and mutation; these stochastic processes become more and more important as the temporal scale increases. In addition, chance events have been shown to be more important in the evolution of traits that are loosely correlated with fitness (Travisano et al, 1995) or are complex, such as growth rate (Marks, C. et al., in press), and have been shown to play a large role in determining adaptation to

changing CO_2 levels in laboratory cultures (Collins et al., unpublished). These findings are especially relevant to phytoplankton responses to rising CO_2, since the carbon concentrating mechanism is a complex trait, and becomes even more so when interactions with other metabolic pathways are considered.

A tacit assumption that is often made when scaling up in time is that the same constraints operate over all timescales of interest. In this case, anything that limits the range of responses of a population to rising CO_2 can be seen as a constraint. At short time scales, constraints are physiological. Over longer time scales, standing genetic variation limits adaptation, while over very long time scales, mutational supply, as well as physical and historical constraints become important. When thinking about population responses to increasing CO_2 by drawing on the wealth of information about physiology, biologists take a bottom up approach to understanding responses, which assumes that the constraints on adaptation are described by limits to changes in induction of a carbon concentration mechanism, the extent of calcification, respiration rates, the production of external polymers, etc. At the other extreme, evolutionary biologists may take a top down approach, arguing that adaptation is limited primarily by access to beneficial mutations. While both of these approaches are useful, they need to be integrated in order to realistically assess what determines how phytoplankton respond to changes that will occur over several decades. It is also important to question what using the information gained by examining each time scale can and cannot tell us. For example, it is reasonable to predict that CO_2 mediated ocean chemistry changes will lead to a shift from coccolithophores to diatoms. This, however, does not tell us whether the diatom populations of the future will fix carbon in the same way as

do contemporary populations, in other words, it does not take into account evolutionary change within taxa as well as displacement of one taxon by another.

Efforts to understand and quantify phytoplankton responses to increasing global CO_2 have recently been undertaken in many different disciplines, each with their own set of assumptions about time scale. Though our understanding of phytoplankton responses to increasing CO_2 has improved dramatically over the past two decades, we still lack important information at every temporal level. In addition, there is little direct treatment of how the rate of environmental change affects adaptive outcomes in either the experimental or modeling literature. At this point, our ability to scale up from experiments in order to provide reliable long-term and large-scale estimates of responses to CO_2 enrichment is extremely limited, though there is a large amount of published data. Efforts to integrate processes that happen on different spatial and temporal scales are necessary in order to draw a realistic picture of future phytoplankton population, and will require collaborative work between disciplines in addition to the current research being done within each area of biology.

Box 1

Time scales of study:

Physiology: Studies the induction of a short-term response, also called an acclimation response. Acclimation may involve the induction or repression of genes already present, and may or may not require the synthesis of new proteins, but does not involve any genetic change. A common physiological response in microalgae to an increase in CO_2 is to decrease the affinity of CO_2 uptake. Acclimation responses of a population do not involve an appreciable change in gene or allele frequencies in the population. Acclimation occurs within a single generation over times ranging from seconds to hours; most experiments here allow about 1 day for acclimation.

Competition (population genetics): These studies involve a shift in the genetic composition of a group, where a more-adapted type replaces a less-adapted type following a change in the environment. Here, the more-adapted type is already present in the population at some frequency, such that no novel genetic change is required, and the final genotype or phenotype of the group will fall within the range of the original group. This can involve a change in genotype frequencies within a population, or the replacement of one taxon by another. This can occur over only a few generations; the rate of change depends on the genetic variance for fitness present in the base population.

Evolution: Studies long-term responses, often called adaptive responses. Adaptive responses involve a change in the genetic composition of a group by the introduction of novel genotypes/phenotypes via mutation and natural selection, such that the final genotype or phenotype of the group may fall outside the range occupied by the ancestor. Adaptive change

occurs over hundreds or thousands of generations in laboratory experiments. In experimental evolution studies, acclimation and sorting occur very early in the experiment or assay and are considered to be an environmental response; the genetic or evolutionary response reported refers to the difference between control or ancestral populations and evolved populations that is attributable to genetic change.

Figure legends

Figure 1: Photosynthetic O_2 evolution rates for three different microalgae acclimated to 360ppmv CO_2 (solid line) and 1800ppmv CO_2 (dashed line). Expected rates were calculated using the standard Michaelis-Menten formula from data in Rost et al., 2003. *E. huxleyi* shows slower saturation of photosynthesis, and so has a larger response to CO_2 enrichment than do the other two microalgae.

Figure 2: Organic carbon pump and carbonate counter pump. Modified from Zondervan, 2001.

Figure 3: Photosynthetic O_2 evolution rates per cell in *Chlamydomonas reinhardtii* after 1000 generations of growth at either high (1050ppm) or ambient (air) CO_2. Symbols signify conditions of selection (Ambient, triangle; High, circle) and assay (Ambient, open; High, filled). The difference in rates between the Ambient- and High-selected lines at a given level of CO_2 represents an evolutionary response. The difference in rates between different levels of CO_2 within either Ambient- or High-selected lines is an environmental (acclimation) response. Reproduced from Collins and Bell, 2004.

Figure 4: The fixation of beneficial mutations in a large population following sudden or during gradual environmental change. Fitness can be absolute or relative. The length of the arrows signifies the effect of the beneficial mutation being fixed.

Figure 5: The scenario being considered. The present wild-type is allele *i*. The *i*-1 alleles to the right are more fit than wild-type, and the many alleles to its left (not shown) are less fit than wild-type. The fitness spacings between the alleles are labeled Δ_1, Δ_2, Δ_3. From Orr, 2002.

Figure 1

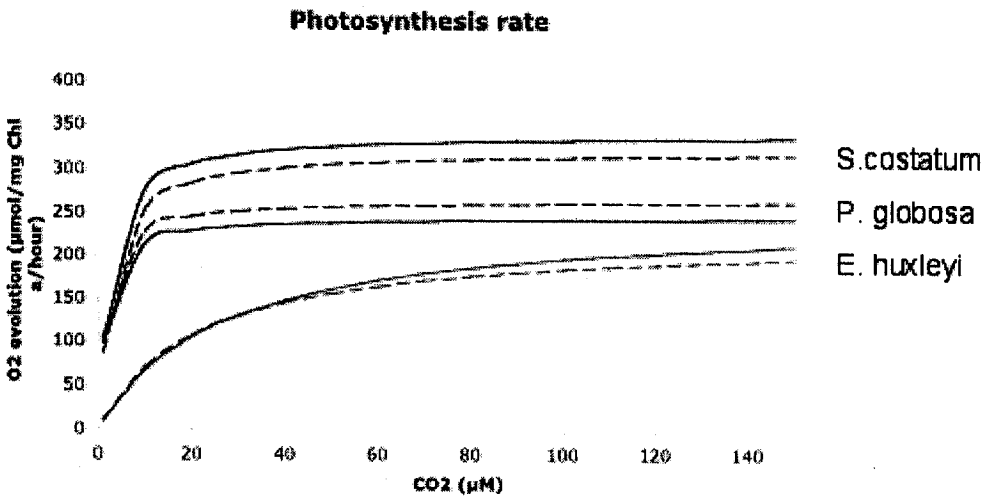

Photosynthesis rate
O2 evolution (µmol/mg Chl a/hour)
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
CO2 (µM)
S.costatum
P. globosa
E. huxleyi

Figure 2

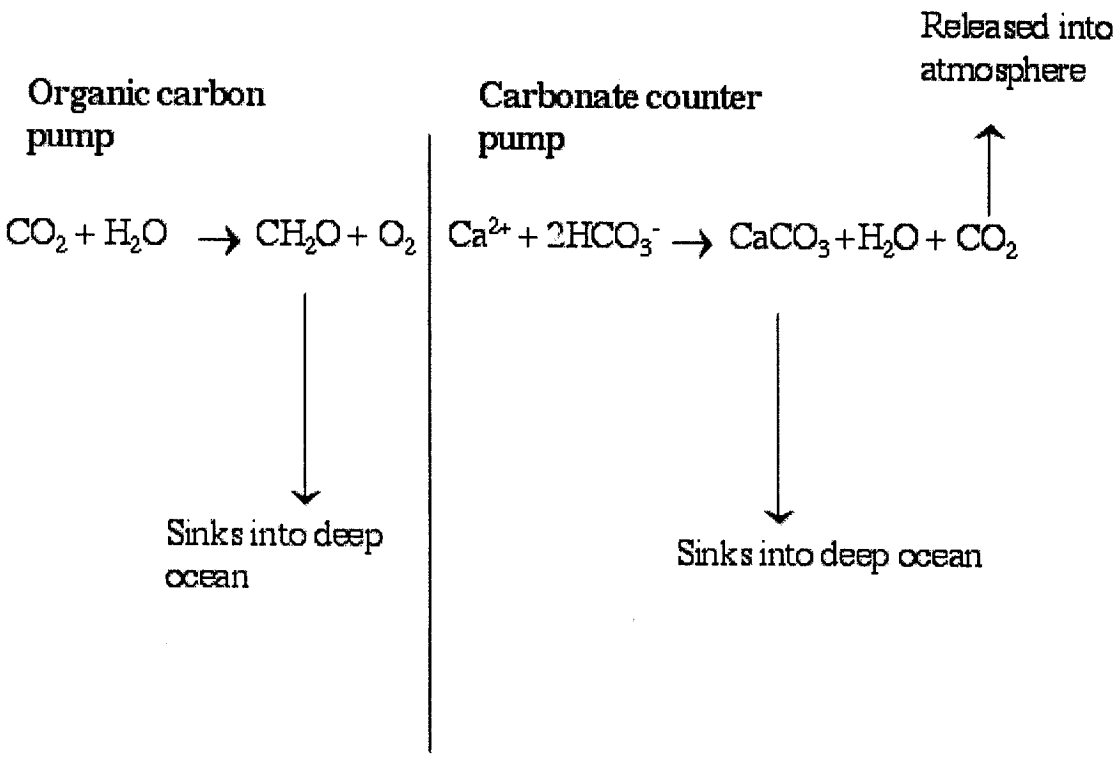

Released into atmosphere
Organic carbon pump
Carbonate counter pump
$CO_2 + H_2O \rightarrow CH_2O + O_2$
$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + H_2O + CO_2$
Sinks into deep ocean
Sinks into deep ocean

Figure 3

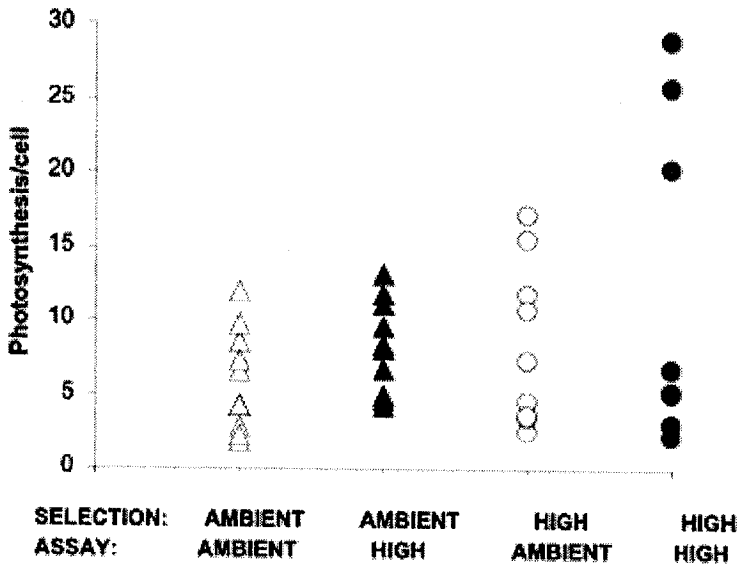

30
25
20
15
10
5
0
Photosynthesis/cell
SELECTION: AMBIENT AMBIENT HIGH HIGH
ASSAY: AMBIENT HIGH AMBIENT HIGH

Figure 4

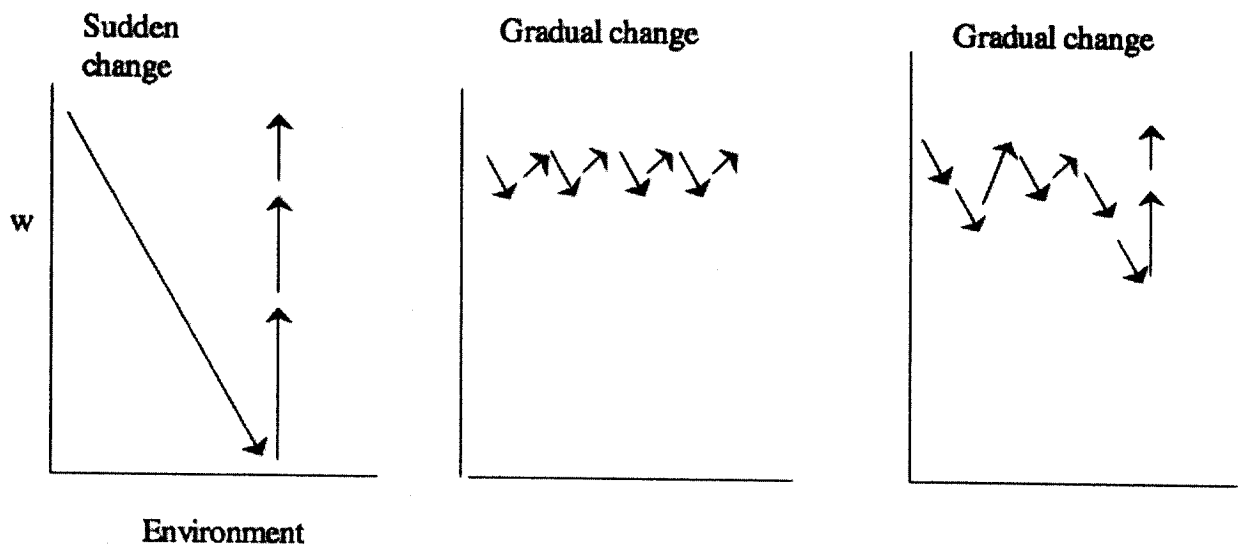
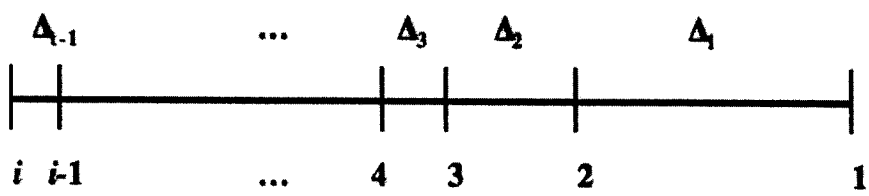

Sudden change
Gradual change
Gradual change
w
Environment

Figure 5



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Appendix: Version of section that will be submitted for publication

Physiological responses to elevated CO_2

The most widely-used definition of phytoplankton responses to changing CO_2 is a physiological one. Physiological responses describe immediate changes in the rates and affinities of carbon uptake and fixation within generations, or changes in population growth rates over short periods of time. It is important to note that a large proportion of physiology studies are designed to explain the current responses of phytoplankton to short-term fluctuations in CO_2, for example over the course of a bloom, or to characterize how inorganic carbon is taken up, and are not meant to be predictive over longer time scales. Experiments have been carried out in both laboratory and natural populations and have been extensively reviewed (Raven, 1991; Beardall et al., 1998; Riebesell, 2005), so are not described in detail here.

Increases in CO_2 lead to decreased affinity for inorganic carbon, sometimes accompanied by a shift from bicarbonate to CO_2 uptake. In summary, most phytoplankton respond to carbon limitation by inducing a carbon concentrating mechanism, which uses a series of carbonic anhydrases to increase the concentration of CO_2 near Rubisco (Moroney and Somanchi, 1999), the enzyme that catalyzes the first and rate-limiting step in photosynthetic carbon fixation. The carbon concentrating mechanism is an inducible system that requires protein synthesis and energy for the active transport of inorganic carbon (Sültemeyer, 1998; Badger et al., 1998; Badger and Spalding, 2000; energetic costs in Raven et al., 2000; Beardall and Giordano, 2002). Both the affinity and inducibility of the carbon concentrating mechanism vary between species (Burkhardt et al., 2001; Colman et al., 2002; Badger et al., 2002; Rost et al, 2003). When CO_2

becomes more abundant, the affinity of the CCM is often reduced, presumably to avoid paying an unnecessary cost of protein synthesis and active uptake. Since some microalgae also actively take up bicarbonate, increases in CO_2 concentration can allow microalgae to shift from bicarbonate to CO_2 uptake; this is thought to be because once CO_2 is abundant, it's uptake requires less energy than does bicarbonate uptake (van Hunnik et al., 2002; Tortell et. al. 2002; Burkardt et al., 2001; Rost et al., 2003). Changes in CCM activity at elevated CO_2 is a highly variable but well-studied response in pure cultures.

A carbon concentrating mechanism that buffers the carbon fixation machinery from changes in external carbon levels should result in phytoplankton whose growth rates are insensitive to increases in CO_2 (Tortell and Morel, 2002; Cassar et al., 2004). However, phytoplankton are usually responsive to CO_2 enrichment in some way (Clark and Flynn, 2000; Beardall et al., 1998). In some cases, CO_2 enrichment has been shown to increase primary productivity in natural populations of phytoplankton (Hein and Sand-Jensen, 1998). In other cases, there is no effect of CO_2 on primary productivity, but there is an effect on phytoplankton biochemistry or physiology such as changes in calcification, nitrogen use, or the production of extracellular polymers (Tortell et al., 2000; Riebesell, 2004; Engel et al., 2005). Though many phytoplankton may show an absence of a short-term growth response to CO_2 enrichment, there is ample evidence that increases in CO_2 correlate with physiological change in phytoplankton, and that many of these physiological changes have the potential to affect interactions between phytoplankton as well as the physical environment should they be sustained over long periods of time.

In addition to laboratory experiments, physiology experiments measure the effects of CO_2 enrichment on natural communities, either in bottles or in ocean enclosures over the course of a bloom. This allows biologists to better interpret the results of laboratory experiments, which typically use high-density monospecies cultures, as well as to evaluate the effect of changes in CO_2 in combination with other associated abiotic changes, such as carbonate chemistry. It also incorporates feedbacks between biotic and abiotic systems that occur over several days. Mesocosm experiments conducted in outdoor seawater enclosures studying the effects of CO_2 enrichment found that *E.huxleyi* blooms were sensitive to CO_2 increases, and reported increases in instantaneous growth rate, though not in total biomass. Decreases in calcification and preferential loss of carbon over other nutrients were also seen (Engel et al., 2005). In an earlier study using bottle incubations over several days, no increase in growth rate or species composition was seen in response to CO_2 enrichment, though short-term carbon fixation rates differed between CO_2 treatments (Tortell et al, 2000). These experiments are important because they demonstrate that even though natural assemblages of phytoplankton are not thought to be CO_2 limited, CO_2 enrichment still causes changes in characters that may be correlated with fitness, which would allow natural selection to act on these populations.

Physiology-based predictions about future phytoplankton populations are based on changes in relative fitness that may occur as a result of a sustained or predictably changing physiological response. The main predictions that result from physiology studies address questions of if and how CO_2 enrichment will affect a given species and often speculate about how this in turn will affect larger scale processes such as competition or abiotic feedbacks. In terms of methods and interpretation of data, one representative example of using physiological data to speculate about

the properties of future populations and their effect on global CO_2 levels by Tchernov et al. used microalgae grown in artificial seawater. In this study, CO_2 and bicarbonate fluxes were measured in several different microalgae grown in either artificial or filtered seawater, containing current amounts of dissolved inorganic carbon. Under the conditions of the experiments, sustained CO_2 efflux occurred along with photosynthetic CO_2 fixation. Based on measurements of contemporary microalgae adapted (and acclimated) to current conditions, the authors suggest that future marine microalgae may constitute a source of CO_2 rather than a sink. In this case, the behavior of contemporary populations under contemporary conditions is directly scaled up to make a prediction about future populations under different conditions. A second example of a physiology-based prediction relies on acclimation responses to elevated CO_2. This has been used to suggest that species that are carbon-limited will benefit most from increasing CO_2 levels (Rost et al., 2003). Here, cultures were acclimated to a given level of CO_2 for at least three days, after which photosynthesis and inorganic carbon uptake rates and affinities are measured. (Figure 1) Here, photosynthesis is not saturated at lower CO_2 levels in coccolithophores, so it is predicted that they would experience the largest change in relative fitness under higher CO_2 conditions, since they will be able to increase CO_2 uptake and photosynthesis in response to rising CO_2.

Physiology-based predictions are based on acclimation responses, and have underlying assumptions that the basic physiology of the species being studied will remain unchanged (or behave predictably) over long periods of time, both in that induction or repression of various processes will be more or less sustained, and that no substantial genetic change affecting the response at the population level will occur over the time scale of interest. As will be discussed,

competition and experimental evolution experiments can be used to directly test these assumptions.

Linking section 2

Since there were no published experimental studies on the evolutionary responses to elevated CO_2 in phytoplankton, we designed one. This study differs from other published work on plant or phytoplankton responses to elevated CO_2 in several respects. First of all, it spans roughly 1000 generations, which is about 100 times longer than other long-term experiments investigating genetic responses to elevated CO_2 in plants. Secondly, CO_2 is increased gradually, rather than abruptly being doubled or tripled at the beginning of the experiment. Third, only CO_2 levels are changed such that the evolutionary response to elevated CO_2 can be disentangled from changes in pH, temperature, or community composition. The intention of this experiment was to investigate if increasing CO_2 alone could drive evolutionary change in microalgae.

Previous work describing evolutionary responses to elevated CO_2 has been done in multicellular plants, partly through experiments spanning several generations (generally fewer than ten), and partly by the characterization of populations found in naturally occurring CO_2 springs. To the best of my knowledge, there are no published descriptions of microalgal populations from CO_2 springs except for a single study using lichen-associated algae. As reviewed in chapter 1, almost all work done on phytoplankton responses to elevated CO_2 measures an acclimation response. Microalgae (phytoplankton) account for roughly half the photosynthetic carbon fixation on earth, and have sufficiently large population sizes and short generation times to evolve in response to elevated CO_2, yet there was little experimental data on what this response could be. An obvious way to remedy this was to carry out a selection experiment.

Chapter 2: Phenotypic consequences of 1000 generations of selection at elevated CO_2 in a green alga

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Estimates of the effect of increasing atmospheric CO_2 levels on future global plant production rely on the physiological response of individual plants or plant communities when exposed to high CO_2 [1-6]. Plant populations may adapt to the changing atmosphere, however, such that the evolved plant communities of the next century are likely to be genetically different from contemporary communities [7-12]. The properties of these future communities are unknown, introducing a bias of unknown sign and magnitude into projections of global carbon pool dynamics. We report the first long-term selection experiment to investigate the phenotypic consequences of selection for growth at elevated CO_2. After about 1000 generations, selection lines of the unicellular green alga *Chlamydomonas* failed to evolve specific adaptation to 1050 ppm CO_2. Some lines, however, evolved a syndrome involving high rates of photosynthesis and respiration, combined with higher chlorophyll content and reduced cell size. These lines also grew poorly at ambient levels of CO_2. We tentatively attribute this outcome to the accumulation of conditionally neutral mutations in genes affecting the carbon concentration mechanism.

Plant growth depends on CO_2 concentration[1,2] and atmospheric concentrations are expected to rise from current levels of about 400ppm to between 700 and 1000 ppm during the next century[3]. In response, global plant productivity in forests[4], grasslands[5], agroecosystems [6] and other ecosystems is expected to increase. Projections of future net primary productivity are complicated by synchronous changes in temperature and other factors, but most models predict increases in the land-atmosphere and ocean-atmosphere fluxes from current values of > -2 PgC/year to about –5 PgC/year[3]. This process is likely to be complicated by shifts in the species composition of plant communities[7], and more fundamentally by evolutionary changes within

plant populations. In the very long term, this may involve the extinction of some groups and the radiation of others[8], but within a few hundred generations most plant populations may adapt to the increased supply of inorganic carbon. Selection experiments with plants have demonstrated a variety of responses[9-12], but have been limited to fewer than ten generations. The long-term response to selection and the properties of populations adapted to elevated CO_2 remain unknown, and constitute an important limit on our ability to predict future plant productivity.

We used a microbial model system in which large population size and short generation time make it possible to evaluate evolutionary change caused by the spread of novel mutations over hundreds of generations. *Chlamydomonas reinhardtii* is a unicellular eukaryotic green alga that has been extensively used to study the physiology and genetics of photosynthesis[13]. It possesses a carbon-concentrating mechanism, which increases the concentration of CO_2 near the active site of Rubisco, in common with most other eukaryotic microalgae that have been studied[14]. We set up 10 isogenic selection lines from each of two ancestral genotypes, half being grown at ambient CO_2 ("Ambient" lines) and half at a concentration that increased from ambient to 1050 ppm over about 600 generations and was then maintained at this level for a further 400 generations ("High" lines). At least 10^5 cells per line were transferred for 125 transfers in a buffered, nutrient rich media. The history of these lines thus resembles the conditions that photosynthetic organisms are likely to experience during the next century or so, with respect to CO_2 levels alone.

The physiological effect of CO_2 on photosynthesis is expected to be an increase in photosynthesis, causing an increase in growth rate. Photosynthesis in the Ambient lines

increased by about 30% when they were grown at high CO_2 (Figure 1A). This is comparable with the physiological response shown by C3 plants from enhanced carboxylation and reduced photorespiration[15]. The Ambient lines diverged through time so that by the end of the experiment they varied significantly in the rate of photosynthesis ($F_{9,18} = 9.015$, $P < 0.001$), when grown at ambient CO_2. The High lines had normal rates of photosynthesis at ambient CO_2, which increased by more than 50% as an average over all lines at high CO_2. This effect was very inconsistent, however: one group of High lines had low rates and a second group very high rates of photosynthesis at high CO_2 (Figure 1A). This distinction was not related to the identity of the ancestor, and represented significantly more divergence in photosynthesis rates than was seen in the Ambient lines ($F_{1,16} = 10.48$, $P = 0.005$) .

The growth rate of cultures grown at elevated CO_2 was correlated with their photosynthesis rate among the Ambient lines, but not among the High lines (Fig 1B). The physiological effect of CO_2 on photosynthesis was reflected by growth in pure culture, where the maximal rate of increase (Figure 1C) and the limiting density (Figure 1D) of both the Ambient and the High lines are enhanced substantially by high CO_2. There was no indication of a parallel evolutionary response, however: by the end of the selection experiment, the High lines had not become specifically adapted to growth at high CO_2, their growth at high CO_2 being no greater than, and perhaps even somewhat less than, the growth of the Ambient lines. There was nevertheless an indirect response: the growth of some High lines was markedly impaired at ambient CO_2, where two of the lines could scarcely be propagated. This result was supported by the outcome of competition assays in which the selection lines were mixed with standard genetically marked strains and the change in frequency during culture growth recorded (Table 1).

The High lines had considerably lower competitive ability at ambient CO_2, where three of them (including the two with strongly reduced growth in pure culture) were such weak competitors at ambient CO_2 that they were consistently eliminated by the tester strains within 10-15 generations. They were, however, no more successful than the Ambient lines at high CO_2. In short, 1000 generations of selection at high CO_2 had caused no increase in growth at high CO_2 , whereas growth at ambient CO_2 was often considerably reduced.

Photosynthesis is linearly related to dark respiration among lines at ambient CO_2; this relationship is the same for Ambient and High lines (Figure 2A). The same correlation is expressed at high CO_2, but respiration rates are on average greater for the High lines, because some High lines have extremely high respiration rates, although others have normal rates of respiration (Figure 2B). It has been shown in *Chlamydomonas* that post-illumination rates of O_2 consumption provide a good estimate of the rates of respiratory O_2 consumption during the preceding light period[27]. Two of the three High lines which had high photosynthetic rates also had high respiration rates, though these high respiration rates do not appear to account for the lack of increase in growth. Other changes such as increased loss of inorganic carbon or higher internal organic carbon content, could explain why the high rates of photosynthesis observed in some lines did not lead to enhanced growth. The evolutionary response is in the opposite sense to the physiological response, which for elevated CO_2 is to induce lower rates of dark respiration in C3 higher land plants.[2]

Both chlorophyll content and cell size responded to selection at elevated CO_2. The physiological response to increased CO_2 is an increase in chlorophyll a content, seen in both the

Ambient and High lines. In the Ambient lines, the average increase in chlorophyll content per cell is about 28%. The High lines, however, show the same inconsistent effect as with photosynthesis rates (Figure 3A); those lines with very high photosynthesis rates at high CO_2 also have very large increases in chlorophyll content at high CO_2. All lines with elevated photosynthesis rates also have increased chlorophyll a content, but increased chlorophyll a content alone is not necessarily associated with a high photosynthesis rate. The physiological effect of high CO_2 is a marked increase in cell volume, both in Ambient and in High lines (Figure 3B). The High lines have smaller cells than the Ambient lines, however, regardless of the concentration of CO_2. The effect is considerable, amounting to an average reduction of 22% in volume. The evolutionary response is thus of comparable magnitude but opposite sign to the physiological response. The outcome of the evolutionary response is a return to the ancestral cell size in the environment of selection, showing a complete attenuation of the physiological response by the end of the experiment.

In addition to a markedly reduced ability to grow at ambient levels of CO_2, the High lines also had a lower limiting density at high CO_2, suggesting either a lowered affinity for CO_2 or a higher per cell requirement for inorganic carbon, which could be the result of higher internal organic carbon content, an increase in carbon used in respiration, increased organic or inorganic carbon loss from cells, or a combination of these. Since no increase in fitness at high CO_2 was seen, any genetic change that occurred was by definition neutral in the selected environment. One possible example of such conditionally neutral mutations in this system would be CCM mutants. Under ambient CO_2 conditions, *Chlamydomonas* and many other microalgae concentrate inorganic carbon through an energy-requiring process which keeps Rubisco saturated

with CO_2 [16-18]. When the external concentration of CO_2 increased, mutations in the down-regulated or unexpressed CCM genes would be neutral in the high CO_2 environment, but deleterious in the ancestral environment. If the CCM were compromised in some way, the evolved lines would show a decreased affinity for inorganic carbon, resulting in a decreased carrying capacity. A lowered affinity for inorganic carbon without a necessary decrease in steady state photosynthesis rates at high CO_2 is seen in some *Chlamydomonas* high-CO_2 -requiring mutants where components of the CCM were inactivated [19-21]. The knockouts often had little or no effect on growth rates at high CO_2. Similarity with knockout phenotypes provides a direction for further characterization of the evolved lines. We tentatively attribute the outcome of selection in our experiment to the accumulation of conditionally neutral mutations which are deleterious when expressed in more stringent conditions. Conditionally neutral mutations have previously been shown to explain antagonistic indirect responses to selection in microbial selection experiments[22-23].

The main result of our experiment is that were we were unable to demonstrate specific adaptation to high CO_2, because after 1000 generations the High selection lines neither grew faster nor had a higher limiting density than did the Ambient lines in the high CO_2 environment. Instead, the physiological response of all traits measured was attenuated or reversed in at least some of the High lines by the end of the selection experiment. This was most clearly shown by cell size, where the evolutionary effect is the opposite of the physiological effect. This suggests that projecting future change on the basis of current physiological responses may be misleading, and should wherever possible be attempted in conjunction with an empirical knowledge of evolutionary responses. A more fundamental obstacle to precise forecasting is the uncertainty of

the evolutionary response, which is a general feature of selection experiments [24-26]. We observed the evolution of two distinct syndromes with respect to carbon metabolism, defined by the ability to respond physiologically to changes in CO_2. One group of lines showed no change in chlorophyll a content, photosynthesis or respiration rates, and when growing at high CO_2 seems to mimic control cells growing at ambient levels of CO_2. This group shows an attenuation of the environmental response to increased CO_2 in that they show little or no increase in photosynthesis rates or associated characters when CO_2 levels increase. The second group of high CO_2 selected lines was more variable in its responses, but all lines within the group had elevated rates of photosynthesis when grown at high CO_2. This second group of lines fixed CO_2 very rapidly but could not channel the fixed carbon into growth. Two of the three evolved lines with elevated photosynthesis rates also had elevated respiration rates when grown at high CO_2. To our knowledge, these high photosynthesis lines do not correspond to any published phenotypes.

The outcome of our experiment suggests that over the next century many phytoplankton communities will evolve less efficient CCMs through the accumulation of conditionally neutral mutations, and will come to consist of smaller cells with broader ranges in photosynthesis and respiration rates than is currently seen. This would affect global processes by changing the rate of carbon turnover in aquatic and perhaps in terrestrial systems. The experimental system, however, is far from perfectly simulating the phytoplankton communities of oligotrophic ocean systems, still less terrestrial plants. We have described phenotypes likely to result from changes in CO_2 levels alone; how changes in other variables, such as temperature, pH and nutrients modify these phenotypes remains to be seen. Selection experiments in more realistic systems will be necessary to validate the evolutionary response to global atmospheric change.

Methods

Selection experiment. Ten replicate lines were founded from a single clone of M566B (lab isolate), and ten replicate lines were founded from a single clone of CC48 (Chlamydomonas Genetics Center, Duke University). Five replicates from each clone were grown in an increasing CO_2 environment and five replicates from each clone were grown in an ambient CO_2 environment. The ambient CO_2 environment consisted of flasks being bubbled with air containing 430ppm CO_2 for the entire experiment. Lines in the High CO_2 treatment were initially grown in flasks being bubbled with air containing 430ppm carbon dioxide, and CO_2 levels were raised steadily to 1050ppm over the first 600 generations of the experiment. These lines were then grown at 1050ppm CO_2 for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300mL of Suoka High Salt Medium[28] in a phytotron chamber under constant light at 25°C. 1mL (about 10^5 cells) were transferred every 3-4 days for approximately 1000 generations for each replicate line.

Pure culture growth rates and carrying capacities. Pure culture growth rates were measured in 384 well plates containing 90μL HSM per well. Cultures were first acclimated (3-6 days), then diluted and transferred to assay plates. For the two evolved lines that often failed to grow at ambient CO_2, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays. The plates were grown in the same phytotron chamber as above at either 430ppm or 1050ppm CO2. Absorbance of each culture was measured every 24 hours. Carrying

capacities were calculated from the maximum absorbance maintained by a culture. Values given are from independent triplicate measurements.

Competitive fitness assay. Competitive fitness was measured by inoculating 300mL of HSM with equal volumes (approximately equal numbers) of acclimated selection line and a marked strain CC48 arg- (from Chlamydomonas Genetics Center, Duke University). The flasks were grown in the same phytotron chamber as above, bubbled with either 430ppm or 1050ppm CO2. The cultures were sampled every three days and plated on HSM + arginine plates. After colony growth, the plates were counted and then replica- plated onto HSM-only plates. The relative frequencies of marker and selection lines were calculated by difference. Dead (arginine-requiring) colonies were usually visible on the HSM-only plates. In cases where the selection lines were absent on plates, they were assumed to be present just below the detection limit of the assay, and were entered into the analysis as having a frequency of 0.005. Values given are from independent triplicate measurements.

Photosynthesis and respiration assays. Cells were acclimated to the assay environment for 3-6 days. Photosynthetic oxygen evolution and respiration (oxygen uptake in the dark) were measured in a Clark-type oxygen electrode illuminated at 800μmol m^{-2} s^{-1}. Cultures were depleted of oxygen by bubbling with N_2/CO_2 at either 430ppm or 1050ppm CO_2. Net photosynthesis (oxygen electrode output from illuminated cells) was used for analysis. Respiration was calculated from oxygen uptake in the dark immediately following a light period.

Values given represent duplicate measurements Chlorophyll was determined by acetone extraction[28].

Cell measurements. Cells from acclimated liquid culture were fixed and measured under a microscope. Cell volume was calculated based on shape of cells[29].

Selection environment	Assay environment	Competitive fitness ± s.e.	Extinct
High CO_2	High CO_2	-0.682 ± 0.306	0
High CO_2	Ambient CO_2	-1.231 ± 0.325	3
Ambient CO_2	High CO_2	-0.292 ± 0.325	1
Ambient CO_2	Ambient CO_2	-0.386 ± 0.290	0

Table 1 **Competitive fitness.** Fitness of all lines was measured against a common marked strain. Selection and marked lines were inoculated in approximately equal numbers at the beginning of the assay. Fitness is calculated such that a fitness of 0 indicates fitness equal to the marker. There is a marginally significant effect of selection on competitive fitness (ANOVA $F=3.92$, $p=0.056$). When a selection line was completely outcompeted by the marked strain by the first time point, it was assumed to be present at the limit of detection (1/200); the number of lines becoming "extinct" is recorded in the final column.

Figure Legends

Figure 1 Response to selection at elevated CO_2. Symbols designate conditions of selection (Ambient, triangle; High, circle) and assay (Ambient, open; High, filled).

(A) Photosynthesis rates measured as O_2 evolution per cell.

(B) Relationship between growth rate and photosynthesis at high CO_2 for Ambient lines ($P = 0.038$) and High lines (ns).

(C) Pure culture growth rates. All values are calculated relative to the average growth rate of Ambient lines growing at ambient CO_2. Lines show increased growth rates at high CO_2 ($F = 33.6$, $P<0.0001$).

(D) Limiting densities. All values are calculated relative to Ambient lines growing at ambient CO_2. High lines have significantly lowered carrying capacities than do Ambient lines (effect of selection $F = 5.1$, $P = 0.03$; effect of assay $F = 32.5$, $P < 0.0001$).

Figure 2. Relationship between photosynthesis and respiration rates at ambient (A) and high (B) CO_2. Photosynthesis and respiration rates are measured as O_2 evolved/sec/cell and O_2 consumed/sec/cell, respectively. Two High lines which failed to grow at ambient CO_2 were excluded. Each point represents independant duplicate measurements of a single line +/- SE.

Figure 3: Correlated responses to selection at elevated CO_2. (A) Chlorophyll a content per cell. (B) Cell volume. Each point represents average cell volume in μm^3 of a single replicate line. Averages were calculated from measurements of approximately 100 cells. Two lines where single cells could not be accurately measured because of clumping were excluded.

Figure 1

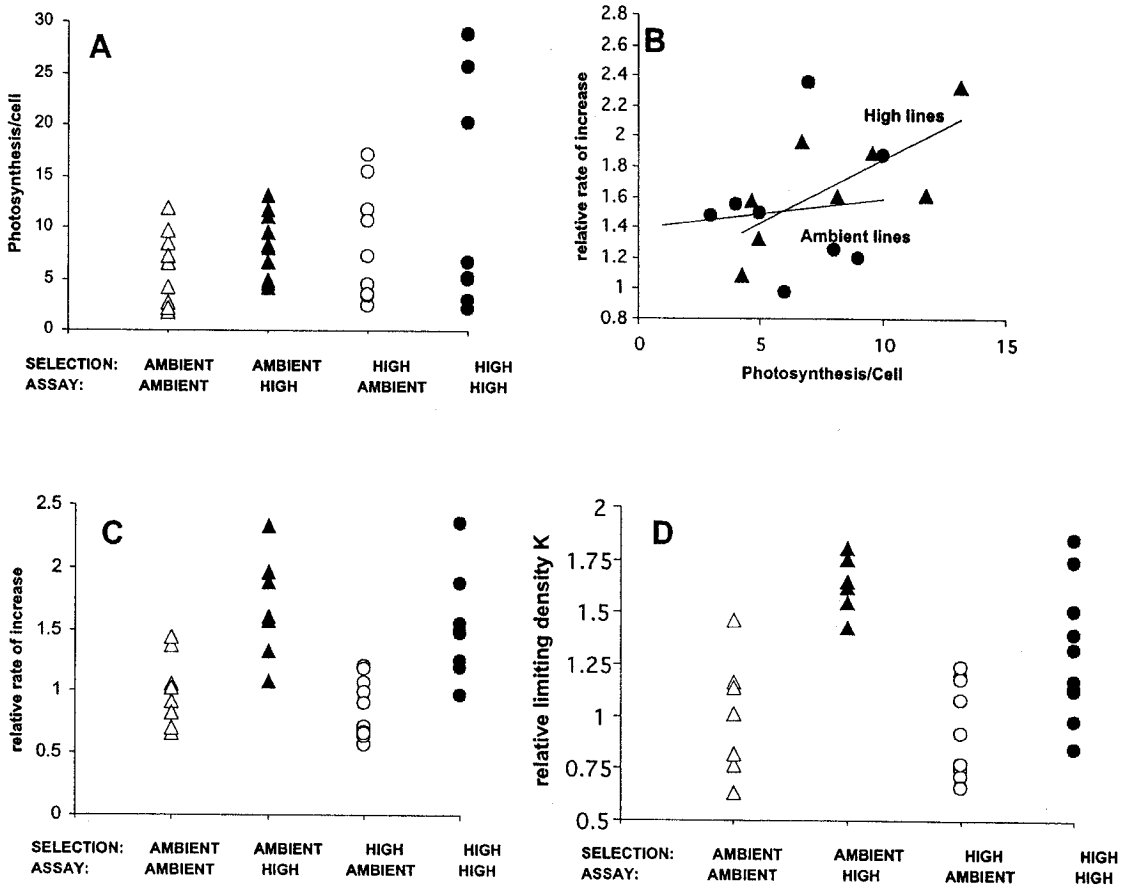
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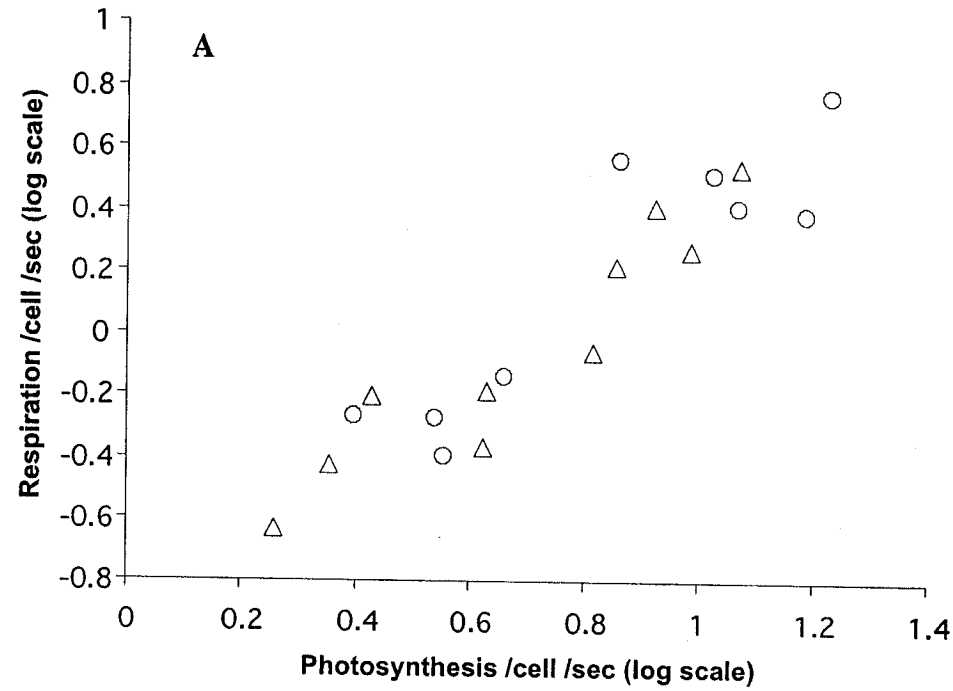
Figure 2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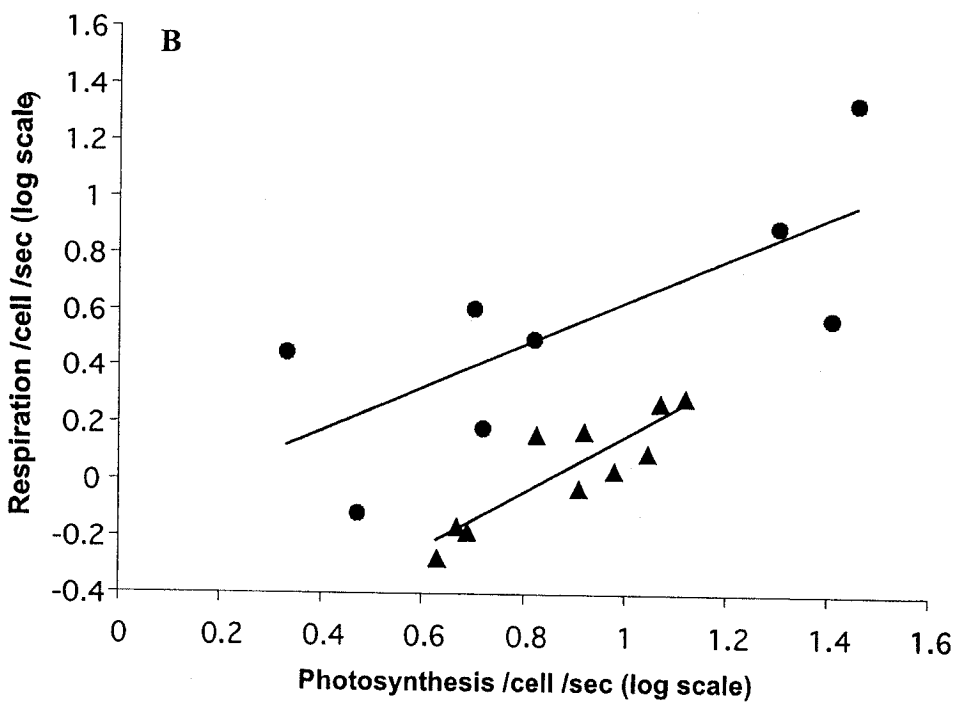
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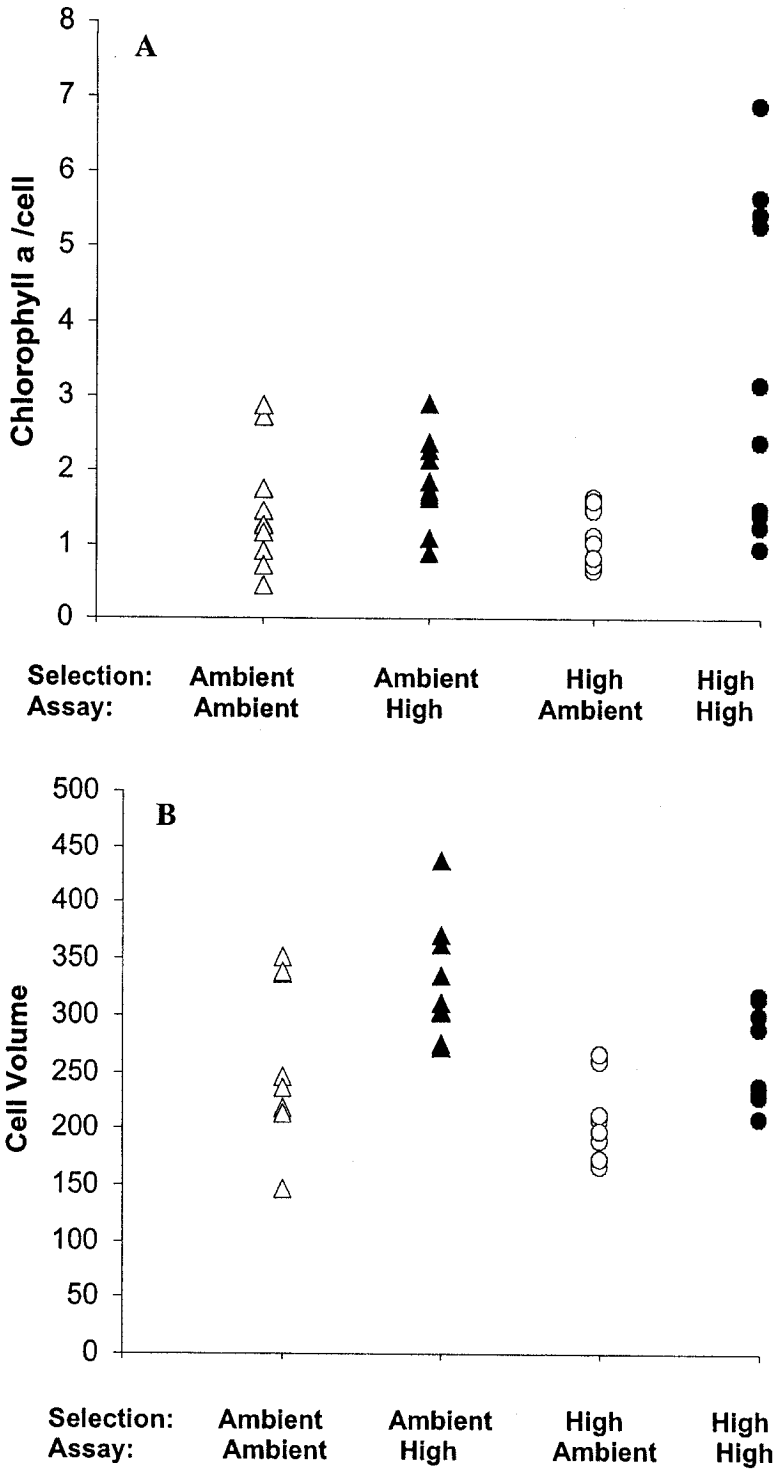
Photosynthesis /cell /sec (log scale)

Figure 3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400
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500
Selection:
Assay:
Ambient
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Appendix

There is no significant effect of ancestor on growth rate (nested ANOVA $F_{1,26}=0.96$, $p=0.34$) or limiting densities (nested ANOVA $F_{1,26}=3.09$, $p=0.09$). In the case of limiting density, the low p value is due to the lines that fail to grow reliably in air, which include 2 lines from one ancestor and one line from the other ancestor. Including the specific ancestor in any of the physiological assays does not appreciably change the results, as is indicated in the manuscript on page 69.

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Linking section 3

The most striking result in Chapter 2 was that there was no direct response to selection at elevated CO_2, but some of the high-selected lines failed to grow at ambient CO_2. The high-selected lines were able to fix CO_2 when it was abundant, but sometimes failed to grow at low CO_2. In addition to this, they had smaller population sizes, suggesting that they had a lower affinity for CO_2. Based on this phenotype, described in chapter 2, we hypothesized that they could not take up inorganic carbon as efficiently as the control lines. Our rationale was that this would have had little effect at elevated CO_2, where the cells could rely more on diffusion, but could lead to starvation when diffusion along with inefficient carbon uptake were not sufficient to saturate CO_2 fixation. In our initial assays, we had not measured CO_2 uptake directly, and so had to conduct additional assays on the high-selected lines. The results of these assays are presented in chapter 3. Chapter 3 provides a more complete physiological characterization of the high-selected lines, and allows for comparison with standard descriptions of the affinity and rate of inorganic carbon uptake in the CCM (carbon concentrating mechanism) literature. This chapter confirms our hypothesis from chapter 2 that the CCM degrades during prolonged growth at elevated CO_2.

Chapter 3: Changes in carbon uptake in populations of *Chlamydomonas reinhardtii* selected at high CO_2

This chapter has been submitted to *Proceedings of the Royal Society of London B* as a manuscript by S. Collins, D. Sültemeyer and G. Bell.

Abstract

Estimates of the effect of increased global atmospheric CO_2 levels on oceanic primary productivity depend on the physiological responses of contemporary phytoplankton populations. It is possible, however, that microalgal populations will adapt to rising CO_2 levels in such a way that they become genetically different from contemporary populations. The unknown properties of these future populations introduce an undefined error into predictions of carbon pool dynamics, especially the presence and size of the biological carbon pump. In order to address the bias in predictions introduced by evolution, we measured the kinetics of CO_2 uptake in populations of *Chlamydomonas reinhardtii* that had been selected for growth at high CO_2 for 1000 generations. Following selection at high CO_2, the populations were unable to induce high affinity CO_2 uptake, and one line had a slower rate of net CO_2 uptake. We attribute this to conditionally neutral mutations in genes affecting the carbon concentrating mechanism. Lower-affinity CO_2 uptake, in addition to smaller population sizes, results in a significant reduction in net CO_2 uptake of about 38% relative to contemporary populations under the same conditions. This shows how predictions about the properties of communities in the future can be influenced by the effect of natural selection.

Introduction

Plant growth depends on CO_2 concentration (reviewed by Urban, 2003), which is expected to increase from current levels of about 400ppm to between 700 and 1000 ppm during the next century (Watson et al., 2001). Oceanic primary production constitutes about 46% of the total primary production on earth (Field et al., 1998) and experiments examining how carbon uptake by microalgae responds to rising CO_2 are needed to understand how oceanic primary production will change in the future. The ocean-atmosphere flux is partially controlled by a biological pump, by which dying phytoplankton sink carbon into deep ocean sediments. Mathematical simulations have estimated that preindustrial levels of CO_2 would have been as high as 460ppm without the operation of such a pump (Sarmiento and Toggweiler, 1984), whereas preindustrial atmospheric CO_2 levels of were around 280ppm (Etheridge et al., 1996). The discrepancy between the a model ocean lacking biologically-mediated carbon fixation and an ocean with biological carbon fixation suggests that biotic sequestration of carbon plays an important role in regulating atmospheric CO_2 levels. It has been suggested that increases in CO_2 may lead to an increase in algal biomass, which would in turn lead to more CO_2 being removed from the atmosphere by these algae. In addition to these ecological responses to rising CO_2, microalgal communities may also adapt to the increased supply of CO_2, resulting in unknown long-term changes to carbon uptake and cycling in oceans.

Most microalgal species respond to CO_2 limitation by the induction of a carbon concentrating mechanism (CCM) (Sültemeyer, 1998; Badger et al., 1998; Badger and Spalding, 2000) The CCM is an inducible system that enables microalgae to respond to extracellular changes in inorganic carbon, and occurs in most microalgal species studied to date (Colman et al. 2002).

The CCM elevates CO_2 concentration in the vicinity of Rubisco, the main carboxylating enzyme in carbon fixation, when carbon is scarce (Moroney and Somanchi, 1999). Conversely, CCM expression decreases at higher levels of CO_2, resulting in lower affinity carbon uptake when inorganic carbon is abundant (Bozzo and Colman, 2000). This allows algae to avoid paying the cost of unnecessary enzyme production and active transport.

Over the long term, changes in CO_2 levels have the potential to affect two different processes connected to phytoplankton growth: carbon uptake and carbon fixation. Most predictions of phytoplankton responses to CO_2 enrichment make the tacit assumption that a functional CCM will continue to exist in phytoplankton and that their basic physiology will remain more or less unchanged as CO_2 rises. If prolonged growth at elevated CO_2 can result in drastic changes to the CCM or to carbon fixation, considerable uncertainty would be introduced into predictions about the outcome of competition between populations, as well as estimates of global carbon pool dynamics.

The presence of a CCM in microalgae buffers the carbon fixation machinery from changes in extracellular inorganic carbon concentrations. In some cases growth and photosynthesis rates are nonetheless stimulated by elevated concentrations of CO_2 within the range of expected global increases (Hein and Sand-Jensen, 1998). In other cases, primary productivity of species or species assemblages of phytoplankton are more or less insensitive to extracellular increases in inorganic carbon (Tortell and Morel, 2002). Even in replicate lines descending from isogenic ancestors, both increases in growth and insensitivity to CO_2 enrichment have been reported (Collins and Bell, 2004). This variability in responses over all time scales makes it difficult to

make general predictions about how phytoplankton populations may respond to elevated CO_2, even when other complicating factors are ignored, such as changes in other nutrients or competition between species.

Variability in the responses of phytoplankton to CO_2 enrichment has led to considerable debate over the role that carbon uptake by phytoplankton plays in the sequestration of anthropogenic CO_2. This has inspired several experiments designed to evaluate the presence and magnitude of a biological carbon pump (Buesseler et al., 2004; Coale et al., 2004; Honda, 2003). In order for anthropogenic CO_2 to be "sunk" by phytoplankton, it must stimulate increases in net primary productivity, either by increasing rates of net carbon uptake or increasing population sizes. Although the response of phytoplankton to changes in CO_2 remains controversial, estimates of increases in primary production of 15-19% in response to reasonable increases in CO_2 have been observed in natural populations in unenriched seawater from the central Atlantic Ocean (Hein and Sand-Jensen, 1998).

We previously used a microalgal model system, *Chlamydomonas reinhardtii*, in order to evaluate changes in fitness caused by the spread of novel mutations over 1000 generations in response to increasing CO_2 (Collins and Bell, 2004). We found that there was no direct response to selection at high CO_2, whereas many of the High selected lines had lowered fitness at ambient CO_2. The lines also had lower maximum population densities at high CO_2, despite normal or increased rates of photosynthesis. From these characters, we suggested that this negative correlated response was caused by the accumulation of conditionally deleterious mutations in the CCM, though this hypothesis was not directly tested. Here, we measure rates and affinities of CO_2

uptake by mass spectrometry to evaluate the hypothesis that prolonged growth at elevated CO_2 can result in the degradation of high-affinity CO_2 uptake. A mass spectrometer was used in order to distinguish between the following hypotheses: that High selected lines were unable to take up carbon, that High selected lines were leaking carbon, or that High selected lines were unable to fix carbon at ambient levels of CO_2, even though it was actively being taken up. Since changes in CO_2 uptake have the potential to affect larger-scale ecological processes, we then use these results to explore the possible effect of evolutionary change on net carbon uptake by algal populations.

Materials and Methods

Selection Experiment. We founded ten replicate lines from a single clone of M566B (lab isolate), and ten replicate lines were founded from a single clone of CC-2344 (Chlamydomonas Genetics Center, Duke University). Five replicates from each clone were grown in a High CO_2 environment and five replicates from each clone were grown in an Ambient CO_2 environment. The Ambient CO_2 environment consisted of flasks being bubbled with air containing 430ppm CO_2 for the entire experiment. Lines in the High CO_2 treatment were initially grown in flasks being bubbled with air containing 430ppm carbon dioxide, and CO_2 levels were raised steadily to 1050ppm over the first 600 generations of the experiment. These lines were then grown at 1050ppm CO_2 for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300mL of Suoka High Salt Medium (Harris, 1989) (pH 7) in a chamber in the McGill Phytotron under constant light at 25°C. We transferred 1mL of culture (about 10^5 cells) every 3-4 days for approximately 1000 generations for each replicate line.

Maximum population densities. Maximum population densities were measured in 384-well plates containing 90μL HSM per well. Cultures were first acclimated (3-6 days), then diluted and transferred to assay plates. For two evolved lines that often failed to grow at ambient CO_2, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays. This may have allowed some backselection of the High selection lines, so that the results presented here are conservative. The plates were grown in the same phytotron chamber as above at either 430ppm or 1050ppm CO_2. Absorbance of each culture was measured every 24 hours. Relative maximum densities were calculated from the maximum absorbance maintained by a culture. The average maximum density obtained by the control cultures growing under ambient CO_2 was arbitrarily given a value of 1. Three independent triplicate measurements were made.

Carbon flux and photosynthesis measurements. We chose three High selection lines which differed in photosynthesis rates, competitive fitness and maximum population densities. CO_2 exchange rates and photosynthetic O_2 evolution in whole cells were measured by mass spectrometry as described by Amoroso et al. (1998), with the following modifications. Cells were acclimated to either 1050ppm CO_2 (high CO_2) or to air (ambient CO_2) for 24 hours. Washed cells were resuspended in a HSM buffered with 50mM bistrisphosphate (BTP) at pH 7.0 at 25°C. Because it is necessary to inhibit the extracellular carbonic anhydrase in order to use this method, 20μM acetazolamide (AZA) was added to the cells. AZA inhibits cell surface carbonic anhydrase (CA), but cannot enter the cell. All selection lines (High and control) as well as the wild-type line had external CA activity. We determined that this concentration of AZA had no effect on intracellular CA, but inhibited external CA in the High and wild-type lines. All our

control lines had evolved insensitivity to AZA and autolysin, presumably due to changes in cell wall composition. Because the method above could not be used on them, the well-characterized wild-type strain (11-32b from Culture collection of algae, University of Göttingen, Germany, described in Amoroso et al.(1998) was used for comparison in CO_2 uptake. Control cultures were used for photosynthetic O_2 evolution, as this does not require inhibition by AZA. CO_2 fluxes were calculated using previously published formulas (Badger et al., 1994). Bicarbonate uptake did not follow Michaelis-Menten kinetics. Net total inorganic carbon uptake was calculated as bicarbonate uptake+ net CO_2 uptake. $K_{0.5}$ (half-saturation constant) and V_{max} (maximum rate of uptake) were calculated by least squares nonlinear regression using Prism 4.0 (GraphPad). We made two or three independent measurements for each estimate.

Net CO_2 uptake calculations. Expected steady-state CO_2 uptake was calculated as maximum population density (relative maximum cell number/population) x (rate of net CO_2 uptake/unit chlorophyll). Rate of CO_2 uptake at a given CO_2 concentration was calculated using the standard Michaelis-Menten formula for a single-substrate reaction from the estimated values of $K_{0.5}$ and V_{max} for cells acclimated to either high or ambient CO_2. CO_2 concentrations used in calculations were the concentration of dissolved CO_2 present in a buffered solution at pH 7 and 25°C at equilibrium given atmospheric concentrations of 1050ppm (high CO_2) or 430ppm (ambient CO_2 during selection experiment). Calculations are given in the Appendix.

Results

Changes in kinetic parameters

All data discussed are for populations. Populations can no longer be considered clonal after 1000 generations of growth and mass transfer, so the error bars represent error + genetic variance in all cases.

Figures1a and 1b show maximum rates of CO_2 uptake for wild-type and High selection lines. The maximum rate of gross CO_2 uptake at a given level of CO_2 is slightly lower in High selected lines than in wild-type lines ($F_{1,20}$=4.83, p=0.04), indicating that the High selected lines do not take up CO_2 as quickly as wild-type. The response of wild-type and High selected lines to changes in CO_2 is the same.

The V_{max} values for net CO_2 uptake varies considerably among High selection lines, but they have a somewhat lower net V_{max} than wild-type at a given level of CO_2 ($F_{1,16}$=4.83, p=0.04). One High selected line (line 10) has a lower maximum rate of net CO_2 uptake than the other High selection lines. This line appears to be unable to prevent CO_2 from leaking out of the cells. The decrease in net CO_2 uptake in the High selection lines is not a consequence of parallel changes in net carbon fixation rates.

Figure 1c shows $K_{0.5}$ for net CO_2 uptake in the wild-type and High selection lines. When grown at high CO_2, wild-type and High selection lines have similar $K_{0.5}$ values, showing that at high CO_2 substrate affinity has not been changed by selection. Wild-type lines show the expected response in $K_{0.5}$ to a decrease in CO_2. There is a selection x assay interaction ($F_{1,14}$=3.57, p=0.08),

indicating that the reaction norm has been changed by selection, such that the High selection lines are unable to induce higher-affinity CO_2 uptake at ambient CO_2.

In terms of the affinity for CO_2, the High selection lines do not differ from the wild-type line when grown at high CO_2, which is consistent with our previous findings that there is no direct response to selection after 1000 generations of selection at high CO_2. The High selection lines have lower affinity for CO_2 when grown at ambient CO_2, however. This is consistent with both the lower maximum population densities and competitive fitnesses of these lines reported previously (Collins and Bell, 2004).

Although the High selection lines were unable to induce high affinity CO_2 uptake, the extent of impairment varies among lines. One of the lines (line 3) has a normal maximum rate of net CO_2 uptake, and is able to prevent CO_2 from leaking out of the cell as effectively as the control and wild-type lines. This line also appears to have a $K_{0.5}$ for net CO_2 uptake that is intermediate between the wild-type and other High selection lines. The other two High selection lines, however, have a lower V_{max} for net CO_2 uptake, indicating that the cells are leaking CO_2, and also appear to have much higher $K_{0.5}$ for CO_2 uptake. Interestingly, line 10, which has the lowest V_{max} and also has a high $K_{0.5}$ was often unable to grow at ambient CO_2, while the other two selection lines always grew.

Bicarbonate uptake does not follow Michaelis-Menten kinetics at pH 7; representative data is shown in Figure 2a. The affinity for total inorganic carbon (bicarbonate + CO_2) is shown in Figure 2b. All lines are capable of taking up bicarbonate. Bicarbonate uptake is important when

inorganic carbon is very scarce, and lowers the $K_{0.5}$ values of total inorganic carbon uptake relative to net CO_2 uptake. Bicarbonate uptake is undetectable when CO_2 is abundant, so V_{max} values are unaffected. Microalgae have been shown to shift from bicarbonate to CO_2 uptake as the amount of CO_2 available increases (Tortell and Morel, 2002), which can occur either as a result of an increase in total carbon or a decrease in pH. Evidence of this can be seen in all lines when the affinity of CO_2 uptake is compared with the affinity of total inorganic carbon uptake. $K_{0.5}$ values for total inorganic carbon uptake are lower than for net CO_2 uptake in all cases ($t=4.38$, $df=7$, $p=0.002$). The wild-type line shows an induction of higher affinity inorganic carbon uptake when CO_2 is lowered, but only one of the three High selection lines can induce higher affinity total carbon uptake. The wild-type shows a 12.5 fold increase in affinity for inorganic carbon, while the High selection line shows a 3.8 fold increase. In this case, the increase in affinity is attributable to increased bicarbonate uptake at CO_2 levels well below ambient. This would not contribute to higher affinity carbon uptake when CO_2 is lowered from high to ambient levels since bicarbonate uptake does not contribute to total inorganic carbon uptake at either high or ambient CO_2. As such, the kinetics of bicarbonate uptake at very low external concentrations of carbon do not appreciably change the conclusions reached using the CO_2 uptake data alone.

Figures 3a and b show V_{max} and $K_{0.5}$ for photosynthetic O_2 evolution. The wild-type and control lines show the same affinity photosynthesis at high CO_2. However, the control line shows higher affinity photosynthesis at ambient CO_2 than the wild-type, which may be a result of selection for growth at ambient CO_2.

There is a significant effect of selection on V_{max} ($F_{1,1}$=16.96, p<0.001), such that the High lines show a reduced maximum rate of photosynthesis. There is no significant effects of selection on $K_{0.5}$ ($F_{1,1}$=3.60, p=0.72), but there is a significant selection x assay interaction ($F_{1,1}$= 9.75, p=0.005), indicating that the reaction norm has been changed by selection. In this case, the High selected lines fail to change the affinity of photosynthesis when CO_2 levels change. However, the High selected lines have higher affinity photosynthesis at high CO_2 than the wild-type and control lines ($F_{1,1}$=9.51, p=0.01). This may partially compensate for the reduced affinity of CO_2 uptake into the cells, as we have previously shown that the High lines do not show a decrease in the actual rate of photosynthetic oxygen evolution at 1050ppm CO_2 relative to the control lines (Collins and Bell, 2004). At ambient CO_2, the High lines are not significantly different than the control line either in terms of affinity or maximum rate of photosynthesis.

The control line has the same response to decreased CO_2 as does the wild-type line (assay x line interaction $F_{1,1}$= 0.32, p=0.60), showing higher affinity photosynthetic O_2 evolution at ambient CO_2 than at high CO_2 ($F_{1,1}$=20.61, p=0.006). One of the High selected lines also shows this response but the other two fail to induce higher affinity photosynthesis when CO_2 is lowered. Most lines show slightly higher maximum photosynthesis rates than would be expected from CO_2 uptake rates alone; this is because of some bicarbonate uptake at low external concentrations of inorganic carbon.

Net carbon uptake calculations Primary production will respond to changes in population size or in the kinetic parameters of carbon uptake in the individuals making up the populations. We combined changes in population density and kinetic parameters to describe how carbon pool

dynamics might change as a result of selection. Net carbon uptake was calculated for each population at a given level of CO_2 using the maximum population size and the kinetic parameters estimated for that population. Table 1 shows the expected relative net CO_2 uptake by contemporary (wild-type) and High selection lines at current and projected levels of CO_2. Both High selection and wild-type lines show a significant increase in net CO_2 uptake when CO_2 is increased ($F_{1,4}$=125.8, $p<0.001$). In all cases, however, the High selected lines have lower net CO_2 uptake than do wild-type lines at a given level of CO_2 ($F_{1,4}$=50.0, $p= 0.002$). On average, the estimates of net CO_2 uptake per population at high CO_2 is approximately 38% less in the High selection lines than in the wild-type lines at high CO_2. In the wild-type populations, net CO_2 uptake increases by 2.4 fold when atmospheric CO_2 increases. In contrast, comparing a wild-type population with a High selection line shows only a 1.5 fold increase between current and projected rates of net CO_2 uptake.

Discussion

The behavior of future phytoplankton populations is of great ecological interest, in part because they have the potential to affect global CO_2 levels. Over the short term, increasing the external concentration of CO_2 can cause phytoplankton to shift from one species of inorganic carbon (bicarbonate) to another (CO_2), accompanied by a decrease in CCM activity (Tortell and Morel, 2002; Burkardt et al., 2001; Rost et al., 2003). The longer-term outcome of growth at elevated CO_2 is seen in our High selection lines, where CCM activity became degraded to the point where high affinity uptake could no longer be induced at ambient CO_2, though it would normally be expressed at ambient CO_2 concentrations in this species (Bozzo and Colman, 2000). In this case, the High selection lines are not being directly compared to an ancestor, however, the control line

could induce higher affinity photosynthesis, suggesting that our control line is not qualitatively different from wild-type. In addition, the control lines originate from a standard unmutagenized lab culture and grow normally in air, which strongly suggests that they have a functional CCM. The complete disappearance of high-affinity CO_2 uptake is a fundamental difference in carbon uptake between wild-type lines of *Chlamydomonas* and the High selected lines characterized here. The induction of higher-affinity CO_2 uptake at ambient CO_2 is a well-documented response in laboratory and natural populations of most microalgae and cyanobacteria, including *Chlamydomonas* (Miyachi et al., 2003). Although experiments with natural populations are needed, our data show that long-term increases in atmospheric CO_2 can degrade CCM induction, leading to greater dependence on CO_2 entry into cells by diffusion. An increased dependence on diffusion at elevated CO_2 may be partially compensated for by higher affinity photosynthesis in some of the High selected lines.

The loss of high affinity CO_2 uptake during long-term growth at elevated CO_2 calls into question the tacit assumption that CCM function will remain unchanged as CO_2 rises. This assumption is often the basis for predictions about the behavior of future phytoplankton populations. For example, the presence of a CCM has been used to suggest that elevated CO_2 alone is unlikely to affect some species (Cassar et al., 2004), as well as to explain interactions between species that may occur at different levels of CO_2 (Tortell, 2000; Riebesell, 2004). Our demonstration that this very basic physiological feature can change in response to elevated CO_2 has major implications for estimations of future population sizes and CO_2 uptake affinities, which directly affect the amount of CO_2 that is fixed by marine populations. Changes in CCM induction also

introduce uncertainty into predictions of relative fitnesses of future populations, which determine the outcome of competition between types.

Previous studies have described CCM knockout phenotypes that resemble our High selection phenotypes (Soupene et al., 2004; Thyssen et al., 2003; Spalding et al., 2002; Suzuki and Spalding, 1988). The results obtained in this study show that the kinetics of CO_2 uptake in the High selection lines have not changed at high CO_2, whereas CO_2 uptake is impaired at ambient CO_2. Since the High selection lines do not have impaired photosynthesis relative to the control, it is unlikely that the High selected phenotypes are attributable to changes in the photosynthesis, such as slower RuBP regeneration. Consequently, we suggest that the High selection phenotypes can be attributed tentatively to conditionally neutral mutations in genes encoding the CCM or related systems during selection at high CO_2. Because the great majority of reported selection experiments show a direct response to selection, it is often difficult to attribute a negative correlated response unambiguously to mutation accumulation rather than to antagonistic pleiotropy (Maclean and Bell, 2002; Turner and Elena, 2000), though studying the dynamics of responses can indirectly demonstrate the presence of mutation accumulation (Funchain et al., 2000). In this case, there was no increase in competitive fitness, growth rate, or maximum sustainable population density in the High selection lines (Collins and Bell, 2004), which strongly suggests that the correlated response is due to mutation accumulation and not to antagonistic pleiotropy. Clear examples of mutation accumulation in large (non-bottlenecked) populations during a selection experiment are rare (Goho and Bell, 2000; Giraud et al., 2001), and are often associated with elevated mutation rates. Our results show that it is possible for mutation accumulation to play an important role in adaptive outcomes, even in large populations

and in the presumed absence of mutators. Further genetic work was not pursued in this study since the underlying genetics have little bearing on characters that are interesting for practical purposes, such as the ability to sink CO_2 into the deep ocean or the ability to compete with other species. In cases such as this one, where the phenotype is of ecological interest, phenotypic convergence between replicate lines can be used to estimate the range of future phenotypes possible. Our finding that a non-adaptive process such as mutation accumulation is likely to affect CO_2 uptake allows a more realistic view of how phytoplankton populations may respond to elevated CO_2.

These results have important implications for projected changes in primary production and carbon sequestration in response to anthropogenic increases in atmospheric CO_2. In order for increased CO_2 to have an effect on carbon sequestration to the deep ocean, it must affect primary production, that is, natural populations must be limited by CO_2 (Riebesell et al., 1993). Though CO_2 limitation of natural populations remains controversial, it has been shown to exist in some laboratory (Riebesell et al., 1993) and natural populations (Hein and Sand-Jensen, 1998; Chen and Dubin, 1994). Our results show that High CO_2 selection lines have a compromised CCM and attain smaller population sizes. As a result of this, the stimulation of net carbon uptake in evolved populations could be significantly less than that projected from the responses of contemporary populations if elevated CO_2 can affect populations through non-adaptive change. Doubts have been voiced over the future possibility of algae acting as a large carbon sink for anthropogenic CO_2 (Hein and Sand-Jensen, 1998; Buesseler et al., 2004; Cassar et al., 2004), even without any sort of evolutionary change in phytoplankton.

The outcome of our experiments suggests that over the next century phytoplankton may evolve a decreased ability to induce high-affinity carbon uptake because of the accumulation of conditionally deleterious mutations in the CCM. The goal of this experiment was not to create a realistic scenario in order to quantify changes in primary productivity and the biological carbon pump. Rather, by changing CO_2 alone in a controlled environment with initially isogenic populations, we have demonstrated the magnitude of bias that evolutionary change can introduce into estimates of future CO_2 uptake by phytoplankton populations. Experiments of this type show that changes in CO_2 alone can change fundamental physiological properties of populations over evolutionary time, in this case disabling the induction of high affinity carbon uptake. This suggests that projections based on the physiological properties of current populations and the assumption that these properties will not change on the scale of hundreds of years may be unrealistic. In addition to evolutionary change within populations, ecological processes such as changes in species composition are likely to make it difficult to predict biotic responses to climate change. Selection experiments in more realistic systems with natural populations are needed to evaluate evolutionary responses to global change.

Appendix 1

Net Carbon Uptake calculations:

1. Rate of net carbon uptake (Vnet)= $V_{max}[CO_2]/(K_{0.5}+[CO_2])$, where V_{max} and $K_{0.5}$ values are those estimated for a particular line at a particular $[CO_2]$. $[CO_2]$ values used correspond to dissolved CO_2 at 1050ppm for High and 430ppm for Ambient, respectively. Values used can be found in Table 2.

2. Net carbon uptake per population = Vnet(relative maximum population size), at a given $[CO_2]$. Maximum population size is relative, with the average of Ambient CO_2 selected lines grown at ambient having an arbitrary value of 1 as described in Collins and Bell, 2004. Values used can be found in Table 3.

Figure captions

Fig.1 A: Maximum rate of gross CO_2 uptake in wild-type and High selection (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A) B: Maximum rate of net CO_2 uptake in wild-type and High selection (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A). C: $K_{0.5}$ of net CO_2 uptake in wild-type and high selected (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A). In all cases, each bar represents averages ± s.e.m. from independent duplicate or triplicate measurements.

Fig. 2 A: Representative bicarbonate uptake data (sqares) from a High selection line. Control and wild-type lines show a similar pattern. Net CO_2 uptake (triangles) is shown to illustrate the relative contributions of CO_2 and HCO_3^- as the concentration of external CO_2 increases. B: $K_{0.5}$ of net total inorganic carbon uptake in wild-type and High selection (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A). Net total inorganic carbon uptake was calculated as (HCO_3^- uptake+ net CO_2 uptake). Each bar represents averages ± s.e.m. from independent duplicate or triplicate measurements.

Fig. 3 A: Maximum rate of photosynthetic O_2 evolution in wild-type, control, and High selection (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A). B: $K_{0.5}$ of photosynthetic O_2 evolution in wild-type, control, and high selected (numbered) lines acclimated to 1050ppm CO_2 (filled bars, H) and air (white bars, A). In all cases, each bar represents averages ± s.e.m. from independent duplicate or triplicate measurements.

Figure 1

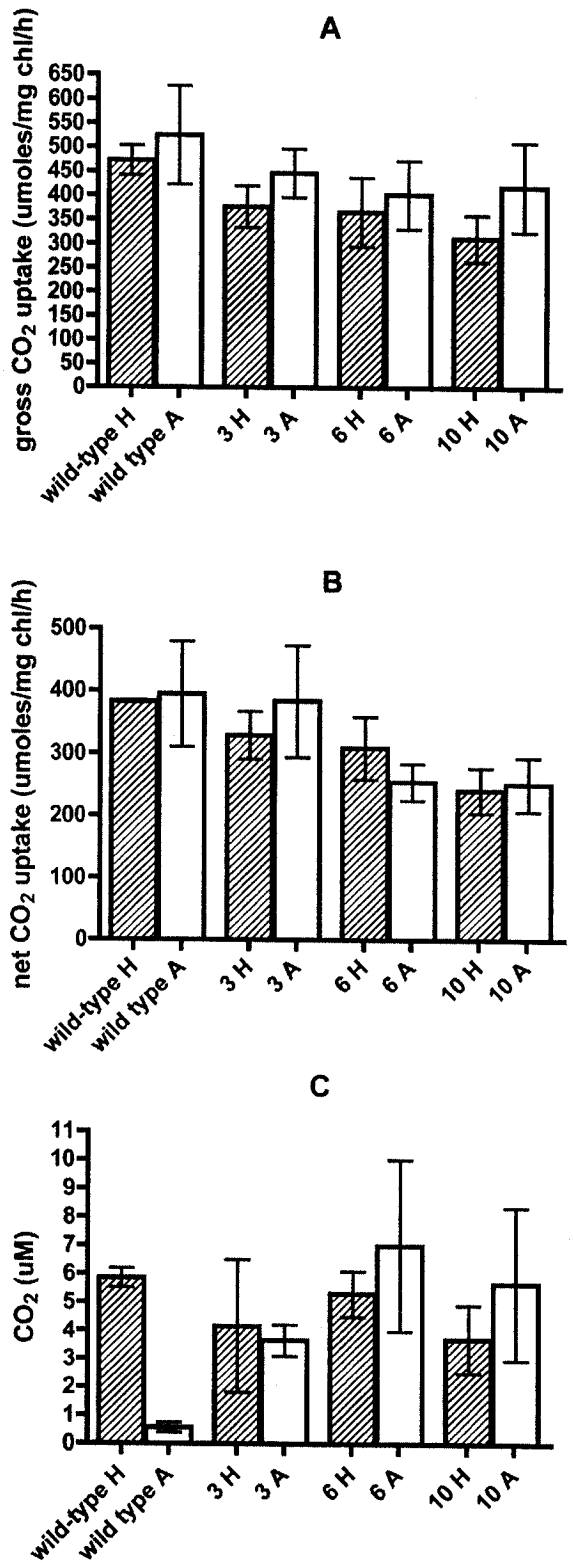
A
gross CO_2 uptake (umoles/mg chl/h)
650
600
550
500
450
400
350
300
250
200
150
100
50
0
wild-type H
wild type A
3 H
3 A
6 H
6 A
10 H
10 A
B
net CO_2 uptake (umoles/mg chl/h)
500
400
300
200
100
0
wild-type H
wild type A
3 H
3 A
6 H
6 A
10 H
10 A
C
CO_2 (uM)
11
10
9
8
7
6
5
4
3
2
1
0
wild-type H
wild type A
3 H
3 A
6 H
6 A
10 H
10 A

Figure 2

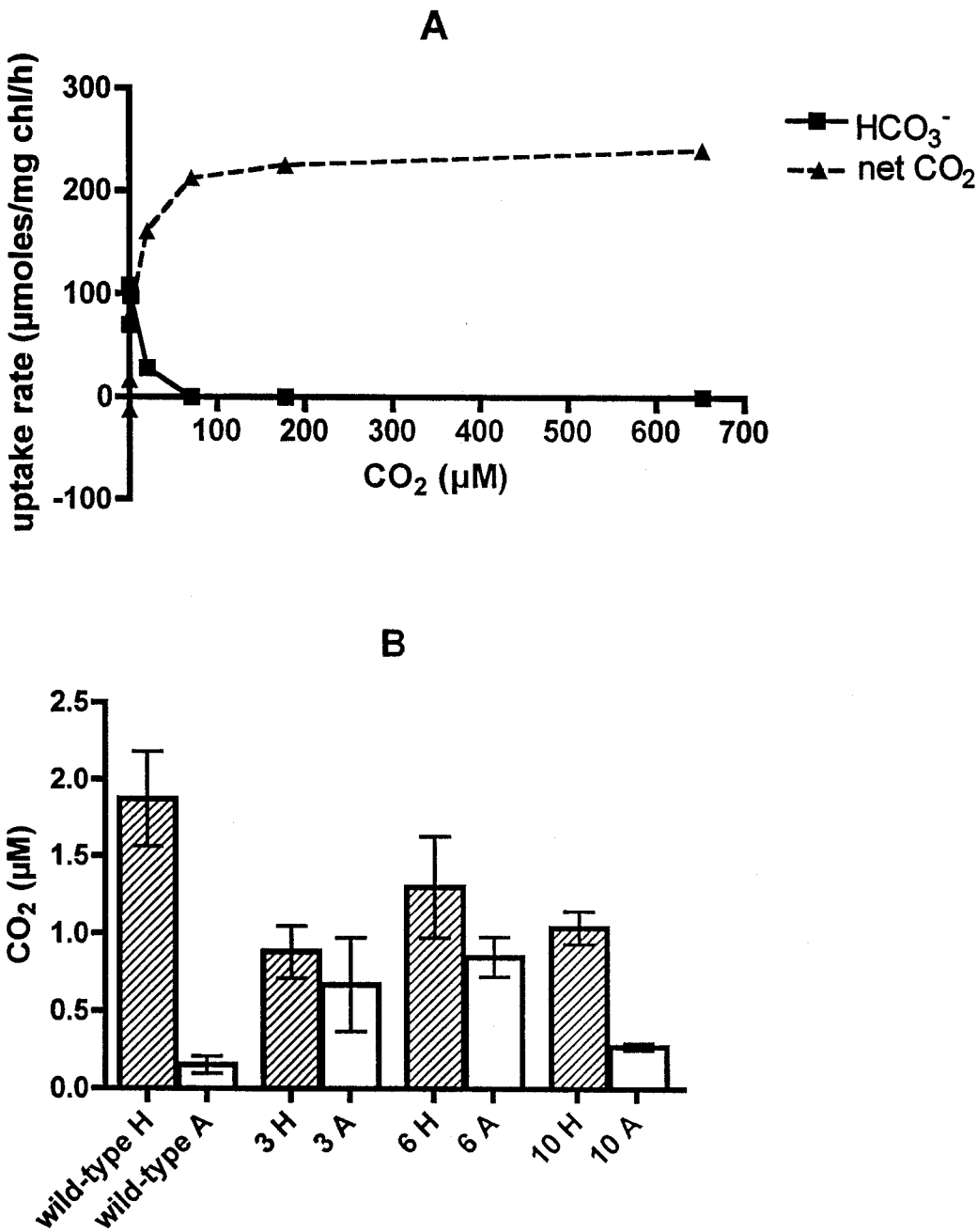
A
uptake rate (µmoles/mg chl/h)
300
200
100
0
-100
100
200
300
400
500
600
700
CO2 (µM)
HCO3-
net CO2
B
CO2 (µM)
2.5
2.0
1.5
1.0
0.5
0.0
wild-type H
wild-type A
3 H
3 A
6 H
6 A
10 H
10 A

Figure 3

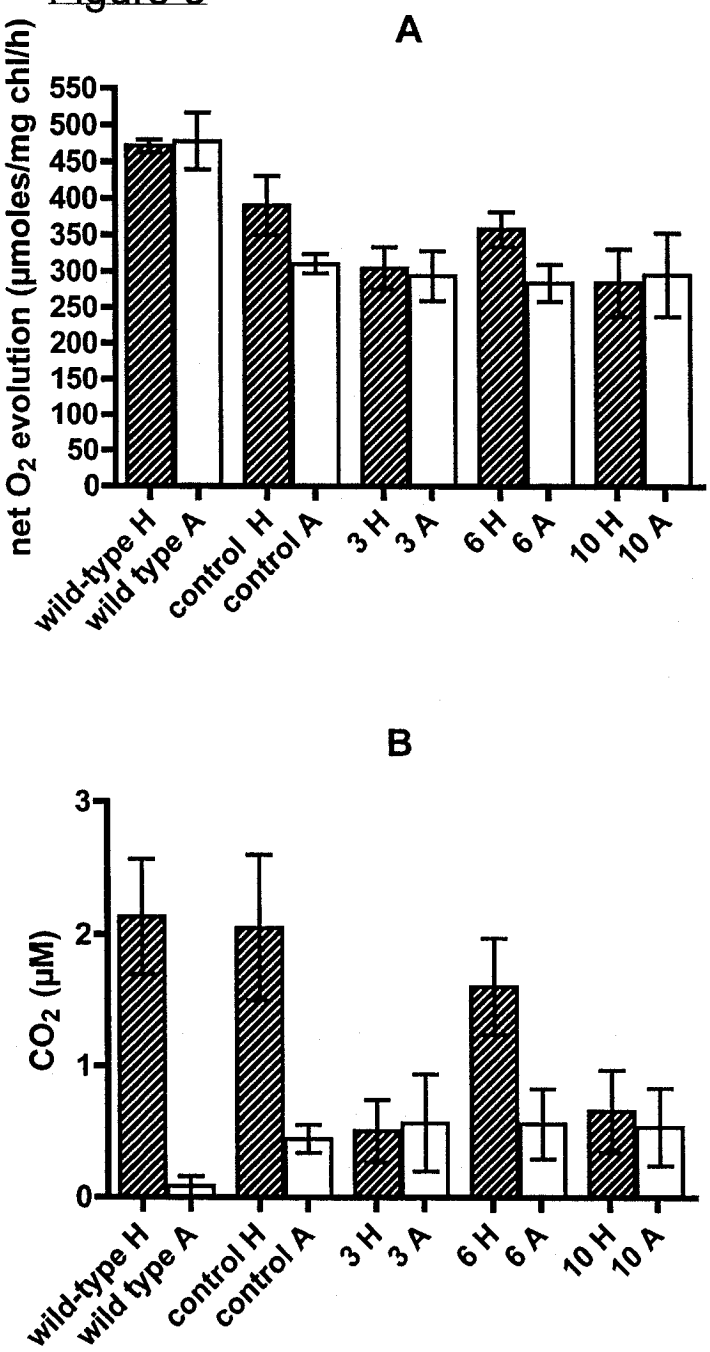
A
net O_2 evolution (µmoles/mg chl/h)
550
500
450
400
350
300
250
200
150
100
50
0
wild-type H
wild type A
control H
control A
3 H
3 A
6 H
6 A
10 H
10 A
B
CO_2 (µM)
3
2
1
0
wild-type H
wild type A
control H
control A
3 H
3 A
6 H
6 A
10 H
10 A

Table 1: Relative rates of net CO_2 uptake per population in wild-type and high selected lines at current and future projected levels (1050ppm) of CO_2. Rates for high selected lines represent the average of the three selected lines studied ± s.e. Rate for wild-type line is estimated from a single wild-type line (11-32b). For calculation procedure, see Appendix.

Tables 2 and 3: Values used for calculation of relative net CO_2 uptake per population in wild-type and high selected lines.

Table 1: Relative rate of net CO_2 uptake per population

	Ambient (current) CO_2	High (projected) CO_2
Wild-type	250	607
High selected lines	57.6 ± 16	375 ± 26

Table 2: Vnet values used:

	V_{net} at Ambient (current) CO_2 (μM/mg chl/hour)	V_{net} at High (projected) CO_2 (μM/mg chl/hour)
Wild-type	250.6	371.9
High selected lines	50.7	286.7

Table 3: Maximum population size values used:

	Relative population size at Ambient (current) CO_2	Relative population size at High (projected) CO_2
Wild-type	1.0	1.63
High selected lines	1.12 (average of High lines)	1.31 (average of High lines)

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Linking section 4

The work in chapter 4 tests the hypothesis that selective history constrains adaptation, and discusses adaptation to changing CO_2 in a more general context than does the rest of the thesis. Up until now this thesis has described the phenotypes resulting from prolonged growth at elevated CO_2 and proposed that no adaptive change occurred because of the accumulation of conditionally neutral mutations in the CCM. This explanation suggests that adaptive change is not possible, at least over a thousand generations. In other microbial selection experiments, the most adaptive change occurs within the first few hundred generations, which supports the interpretation that the ability of *Chlamydomonas* to respond adaptively to elevated CO_2 is somehow constrained, rather than that the experiment was prematurely ended. The description of and explanation for high-selected phenotypes in chapter 3 does not attempt to explain why no adaptive change occurs. It instead describes which other processes occur in the absence of adaptation.

The question of whether selective history constrains adaptation to changes in CO_2 can also help us to gain insight into the current biology of phytoplankton, since CO_2 fluctuates over time scales where adaptation can occur (glacial-interglacial cycles, for example), as well as on shorter timescales where acclimation is more important (over the course of a phytoplankton bloom). Because of this, evolutionary responses to changes in CO_2 may have been optimized over time not to adapt particularly well to any one level of CO_2, but rather to avoid doing particularly poorly at any given level of CO_2.

Chapter 4: Rewinding the tape: selection of algae adapted to high CO_2 at current and Pleistocene levels of CO_2

This chapter has been submitted to *Evolution* as a manuscript by S. Collins, D. Sültemeyer and G. Bell

Abstract

Selective history is thought to constrain the extent and direction of future adaptation by limiting access to genotypes that are advantageous in a novel environment. Populations of *Chlamydomonas* previously selected at high CO_2 were either backselected at ambient levels of CO_2, or selected at levels of CO_2 that last occurred during glaciation in the Pleistocene. There was no effect of selective history on adaptation to either level of CO_2, and the high CO_2 phenotypes were evolutionarily reversible such that fitness in ambient CO_2 returned to values seen in controls. CO_2 uptake affinity improved relative to the ancestor in both ambient and glacial CO_2, though wild-type regulation of CO_2 uptake, which deteriorated during previous selection at high CO_2, was not restored by selection at lower levels of CO_2. Tradeoffs in both CO_2 uptake affinity and growth were seen after selection at any given level of CO_2. Ad aptation to ambient and glacial-era levels of CO_2 produced a range of phenotypes, suggesting that chance rather than history contributes to the divergence of replicate populations in this system.

One of the most influential ideas in evolutionary biology is that of the importance of historical contingency on adaptive outcomes, which is often articulated as the thought experiment of "replaying life's tape" (Gould 1989). Several experiments have demonstrated that replicate populations tend to become convergently adapted, while diverging in terms of underlying characters such as specific mutations, physiology or morphology (Travisano and Lenski 1996; Travisano 1997; Nakatsu et al. 1998; Riley et al. 2001; Teotónio et al. 2002; Yedid and Bell 2002; MacLean and Bell 2003). These have confirmed experimentally that adaptive outcomes tend to be unique at some level, even over relatively short timescales. History contributes to this when an ancestral state constrains future adaptation by limiting the range of beneficial changes that can evolve from some particular starting point. The most fundamental way that this has been shown to occur is by limiting access to beneficial mutations by creating a mutational neighborhood defined by the set of mutants one or a few mutational steps away from the ancestral state (Burch and Chao 2000). The consequence of this sort of constraint is that populations subjected to the same selection regime do not all end up with the same genotype, so that genotypes and phenotypes differ between replicate populations, and some populations may even fail to adapt to a novel environment. For example, it has been suggested that the ability of contemporary plants to adapt to a high CO_2 world is constrained by past adaptation to lower CO_2, as shown by constitutively high levels of Rubisco, Rubisco activase, and carbonic anhydrase (Sage and Coleman 2001; Coleman 2000).

A second reason that replicate populations subjected to the same selection pressure may diverge is that the appearance and spread of novel beneficial mutations is a stochastic process. As a result of finite population sizes and relatively large genomes, only a small fraction of possible

combinations of mutations occur every generation. In addition, beneficial mutations are often lost to chance when rare. This creates, by chance, early differences between replicate populations. These differences constrain further adaptation since epistatic interactions now limit the beneficial states accessible by unit genetic change in a given population. The result is that the order that beneficial mutations fix in is a particular population is idiosyncratic (Muller 1939; Mani and Clarke 1990; Korona 1996). In contrast, a higher degree of convergent genetic evolution has been seen in retroviruses, where genome size is small and population size large, such that the order of fixation of mutations becomes less stochastic (Cuevas et al. 2002). The relative contributions of adaptation, history and chance to microevolutionary outcomes can be quantified using selection experiments using several different ancestors, each used to found several replicate populations. All three factors have been found to contribute to outcomes with chance and history playing a relatively small role in the evolution of fitness and characters closely correlated with fitness (Travisano et al. 1995).

One of the consequences of populations evolving idiosyncratically is that evolution should be irreversible, given that the probability of retracing a specific series of mutational events is small, as is the chance of serendipitously ending up at the ancestral genotype by some other route. However improbable the reversal of evolution is at a genetic level or over very long time scales, several examples of reversible phenotypic evolution have been seen in microevolution experiments (Travisano et al. 1995; Teotónio et al. 2002; Estes and Lynch 2003). Backselection experiments can examine if and how phenotypes are evolutionarily reversible; they can also give important insights into the current biology of a system and allow us to study how fitness is recovered. The reversibility of phenotypes over evolutionary time is of particular interest in

environments that fluctuate very slowly relative to the life cycle of the organism, such as variations in CO_2 and temperature during glacial-interglacial cycles. This forces populations to become repeatedly adapted to two recurring environments over evolutionary time. Though environmental fluctuation at shorter time scales has been shown to cause the evolution of generalist types (Reboud and Bell 1996), it is unclear if fluctuations over hundreds or thousands of generations systematically affect adaptive outcomes or current biology. As with short-term environmental fluctuations, any adaptation to current conditions that completely inhibited adaptation to future conditions would not persist through more than half a cycle of environmental change. Over several cycles, this could result in a system that was selected for long-term evolvability. Adaptation to a fluctuating environment over microevolutionary time scales has been shown to result in a "bet-hedging" strategy, where the lineage that persists over time may not be the one that performs best in the average environment, but rather the one that is able to avoid performing poorly (or going extinct) in the most hostile environment encountered (Gillespie 1973, 1974). This strategy results through repeated reversals of adaptive trends over shorter time scales (Simons 2002). Reverse evolution may also be of interest in practical questions such as the evolution of antibiotic resistant bacteria or in environmental cleanups, involving reductions in concentrations of nutrients or toxins for bacterial, algal and plant populations that have become adapted to pollutants. Reverse evolution and fitness recovery are also important in the conservation of bottlenecked populations where adaptedness may have been degraded by mutation accumulation.

Replicate populations of *Chlamydomonas reinhardtii* selected for 1000 generations at high CO_2 failed to show any adaptive change to the high CO_2 environment, and the among-population

variance of fitness and most related characters increased, suggesting relaxed selection (Collins and Bell 2004). Several of the populations showed reduced fitness in the ancestral environment, and some at least had lost the ability to induce high-affinity CO_2 uptake, a basic and well-characterized process in *Chlamydomonas* and many other microalgae (Collins et al. 2005 unpubl.). Most microalgal species respond to CO_2 limitation by the induction of a carbon concentrating mechanism (CCM) (Sültemeyer 1998; Badger et al. 1998; Badger and Spalding 2000) The CCM is an inducible system that enables microalgae to respond to extracellular changes in inorganic carbon, and occurs in most microalgal species studied to date (Colman et al. 2002). The CCM elevates CO_2 concentration in the vicinity of Rubisco, the main carboxylating enzyme in carbon fixation, when carbon is scarce (Moroney and Somanchi 1999).

In this study, High selected populations were backselected down to ambient CO_2 levels and low (Pleistocene glacial) levels of CO_2. Neither of these environments is completely novel, in that the high-CO_2-selected *Chlamydomonas reinhardtii* (or its predecessor) has experienced them in either the recent or very distant past, but both are environments that the High selected lines were poorly adapted to at the beginning of the experiment. This design allows us to address two questions. Firstly, how does selective history constrain adaptation and the reversibility of evolution? More specifically, how does natural selection reverse loss-of-function phenotypes, such as the high-CO_2-requiring types that resulted from selection at high CO_2?

Methods

Selection experiment: The experimental design is shown in Figure 1. We founded ten replicate lines from a single clone of M566B (lab isolate), and ten replicate lines from a single clone of CC-2344 (Chlamydomonas Genetics Center, Duke University). Five replicates from each clone were grown in a High CO_2 environment and five replicates from each clone were grown in an Ambient CO_2 environment. The Ambient CO_2 environment consisted of flasks being bubbled with air containing 430ppm CO_2 for the entire experiment. Lines in the High CO_2 treatment were initially grown in flasks being bubbled with air containing 430ppm carbon dioxide, and CO_2 levels were raised steadily to 1050ppm over the first 600 generations of the experiment. These lines were then grown at 1050ppm CO_2 for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300mL of Suoka High Salt Medium, pH 7 (HSM, Harris 1989) in a chamber in the McGill Phytotron under constant light at 25°C. We transferred 1mL of culture (about 10^5 cells) every 3-4 days for approximately 1000 generations for each replicate line.

Five of the High selection lines and five of the Ambient selection lines were chosen for selection at decreasing CO_2, such that a broad range of variation in growth and other characters (such as photosynthesis rate) was represented. Each line was split into 4 replicates for each treatment. The replicate lines were grown in separate phytotron chambers at either a Low CO_2 environment (180ppm) or an Ambient CO_2 environment (430ppm). CO_2 levels were controlled as described in Romer (2001). Lines in the Low CO_2 treatment were initially grown in flasks being bubbled with air containing 430ppm CO_2, which was then decreased steadily to 180ppm over 17-26 transfers, or about 150-225 generations. Lines that grew more slowly were transferred less frequently.

Lines in the Ambient CO_2 treatment were grown at 430ppm CO_2 for the duration of the experiment. Several of the High selected lines initially failed to grow at lower CO_2. These lines were re-inoculated several times. In all cases, lines were propagated by serial transfer, and were transferred when they reached a density of approximately 10^5 cells/mL. The format used to refer to each line is Selection(History): for example, a line initially adapted to high CO_2 that was then selected at ambient CO_2 is Amb(High).

Growth assays: Pure culture growth rates were measured in 384-well plates containing 90μL HSM per well. Cultures were first acclimated (3-6 days), then diluted and transferred to assay plates. For High or Ambient selection lines that often failed to grow at lower concentrations of CO_2, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays at lower concentrations of CO_2. This may have allowed some backselection of the lines, so that the results presented here are conservative. The plates were grown in the same phytotron chamber as above at either 180ppm, 430ppm or 1050ppm CO_2. Absorbance of each culture was measured every 24 hours. Relative maximum densities were calculated from the maximum absorbance maintained by a culture. The average maximum growth rate or density attained by the Amb(Amb) lines growing under ambient CO_2 was arbitrarily given a value of 1 in all assays so that the growth rates are comparable across the entire experiment. The direct response to selection was calculated as increase in either growth rate or carrying capacity relative to ancestor in the selected environment.

Sorting response to low CO_2 in ancestral populations: Since some of the High and Ambient selection lines failed to consistently grow at lower concentrations of CO_2, we hypothesized that

they contained at least two types, at least one of which was incapable of growing at lower concentrations of CO_2. If this were true, acclimation cultures inoculated with only high-CO_2-requiring cells would not grow, while those inoculated with either low-CO_2-tolerant cells or a mix of the two types would grow. In order to test whether some of the High and Ambient selection lines contained a mix of low-CO_2-tolerant and high-CO_2-requiring types, the ancestral populations for the Low selected lines were inoculated into HSM and acclimated to the environment they were originally selected in (either high or ambient CO_2). Serial dilutions of the growing cultures were transferred to 384-well microplates, placed under low CO_2 and allowed to grow for 10 days, until all surviving cultures reached maximum population densities. The frequency of High or Ambient CO_2-requiring cells in the ancestral populations was estimated by plotting the number of cells initially present in each well against the proportion of wells that grew at low CO_2 over 10 2-fold dilutions for each ancestral population. The largest population size where no cultures were able to grow was taken as the maximum frequency of types that could grow at low CO_2. For example if no cultures grew at an initial inoculum of 45 cells, but a subset grew with an initial inoculum of 90 cells, then the maximum frequency of cells capable of growth at low CO_2 is 1/45. The limit of detection of this assay is a frequency of 0.5 because cultures of *Chlamydomonas* do not grow reliably from an average inoculum of fewer than 2 cells in this assay. Replicate plates of the 2 lowest dilutions were grown at Ambient CO_2 to test if the diluted populations contained enough viable cells to grow in the ancestral environment.

Carbon flux measurements: CO_2 exchange rates in whole cells were measured by mass spectrometry as described by Amoroso et al. (1998), with the following modifications. Cells were acclimated to either 1050ppm CO_2 (high CO_2), to air (ambient CO_2), or to 180ppm CO_2

(low CO_2) for 24 hours. Washed cells were resuspended in HSM buffered with 50mM bistrisphosphate (BTP) at pH 7.0 at 25°C. Because it is necessary to inhibit the extracellular carbonic anhydrase in order to use this method, 20μM acetazolamide (AZA) was added to the cells. AZA inhibits cell surface carbonic anhydrase (CA), but cannot enter the cell. We determined that this concentration of AZA had no effect on intracellular CA. All our control lines had evolved insensitivity to AZA and autolysin, presumably due to changes in cell wall composition. Because the method above could not be used on them, the well-characterized wild-type strain 11-32b (Culture collection of algae, University of Göttingen, described in Amoroso et al. (1998)), was used for comparison. CO_2 fluxes were calculated using previously published formulas (Badger et al. 1994). $K_{0.5}$ (half-saturation constant) and V_{max} (maximum rate of uptake) were calculated by least squares nonlinear regression using Prism 4.0 (GraphPad). Bicarbonate uptake was found to be negligible at pH 7.0 (data not shown), so only CO_2 uptake is reported here .We made two or three independent measurements for each estimate.

Results

Tempo of adaptation to low CO_2: Several of the ancestral lines often failed to grow at low CO_2, though if enough cultures were inoculated, a subset would grow. This suggested that these ancestral populations were made of several phenotypes, at least one of which was unable to grow at low CO_2. Acclimation to low CO_2 for the growth assays described above lasts for several generations, and may present an opportunity for an initial rapid response to low CO_2 by sorting of the base population. Out of 10 ancestor populations, four showed evidence of an initial rapid adaptation to low CO_2 by sorting. The estimated frequencies of the phenotypes able to grow at low CO_2 in these cultures is listed in Table 1. For some ancestor populations, the frequency of

types able to grow at low CO_2 is listed as >0.5; this is because at or below an average inoculum of 2 cells, it is not possible to tell if a culture fails to grow because it contains high-CO_2-requiring cells or because it contains no viable cells at all.

Direct response to selection: Responses to selection are summarized in Table 2. Figures 2a and b show the maximum growth rates and maximum population densities of lines selected at low CO_2. There is no significant effect of selective history (either High or Ambient selection) on the growth rates ($t=0.49$, $df=12$, $p=0.64$) or population densities ($t=0.64$, $df=12$, $p=0.53$) of the Low selection lines. Low selection lines arrive at the same range of growth rates and population sizes regardless of selective history.

Figure 3 shows the direct response to selection at low CO_2 in lines that were previously selected at either High or Ambient CO_2. There is no effect of ancestor in the Low selection lines on either growth rate ($F_{9,8}=0.47$ and $F_{9,8}=0.94$, combined $p=0.80$) or maximum population density ($F_{9,8}=1.08$ and $F_{9,8}=0.59$, combined $p=0.68$) across all three environments (high, ambient and low CO_2). Since there is no effect of selection history on response or outcome of selection at low CO_2, the data from Low(High) and Low(Amb) lines was pooled for the purpose of analysis here. The mean direct response to selection is positive for maximum population sizes ($t=5.17$, $df=13$, $p<0.001$), and for maximum growth rate ($t=2.04$, $df=13$, $p=0.03$). On average, there is a 2.1 fold improvement over the ancestral population density and a 1.6 fold increase over the ancestral growth rate. Each line had a greater maximum growth rate, maximum cell density, or both, than it's ancestor. Several of the lines with High ancestors failed to show any increase in maximum population size. In cases where there was little or no response, the ancestor already had a high

maximum cell density.

Figure 4 shows the growth rates and maximum population densities of Amb(High) and Amb(Amb) lines grown at ambient CO_2. Amb(High) lines return to the same mean growth rates ($t=0.91$, $df=15$, $p=0.37$) and maximum cell densities ($t=0.92$, $df=14$, $p=0.37$) in their ancestral environment as cultures that were grown at ambient CO_2 for the entire selection experiment, though the between line variance in maximum cell density in the backselection lines is lower than in the Ambient selected lines ($F_{6,8}=14.85$, $p=0.001$).

Indirect response to selection: The expected response to an increase in environmental CO_2 is an increase in growth rate. On average, lines selected at Low CO_2 respond to an increase in CO_2 by increasing growth rate ($F_{2,2}=20.10$, $p<0.0001$) (see Table 2). However, two of the Low-selected lines fail to grow reliably at ambient CO_2, and one fails to consistently grow at high CO_2. The same average result, an increase in growth rate in response to increases in CO_2, was previously reported in both High and Ambient ancestor populations (Collins and Bell 2004), with some High lines failing to grow reliably at ambient CO_2. However, none of the ancestral Ambient lines failed to grow at high CO_2. The appearance of lines unable to grow at levels of CO_2 greater than those they were selected at was only observed in lines selected at Low CO_2.

Changes in CO_2 uptake affinity: Figure 5 shows the changes in net CO_2 uptake affinity in Low and backselected lines. Low lines generally evolve higher affinity net CO2 uptake than their ancestor (paired $t=2.56$, $df=3$, $p<0.01$; mean difference=9.35μM). Amb(High) and Low(High) lines do re-evolve a response for changes between high and ambient CO_2, though this was done

by decreasing affinity at high CO_2 rather than improving affinity at ambient CO_2. For all three Low selection lines measured, a large decrease relative to the ancestor in net CO_2 uptake affinity at High CO_2 was seen (paired t=8.48, df=2, $p<0.01$; mean difference=26.29μM).

Discussion

Our selection experiment was designed to investigate constraints to adaptation to lower concentrations of CO_2. In microbial systems used to study adaptation, sorting of genetic variance for fitness does not occur at the beginning of the experiment, since selection experiments are traditionally founded with clonal cultures. In this case, the Low selection lines were founded from genetically diverse populations. Because of this, adaptation to sudden drops CO_2 experienced by the populations during growth assays occurred in two steps: a rapid sorting step, and a slow adaptive step. In the sorting step, high or ambient CO_2-requiring mutants are replaced by types that are capable of growing at low CO_2. We estimated that phenotypes requiring high or ambient CO_2 exist in at least 4 of the 10 populations used as founders of Low selection lines. This sorting resembles changes in population or species composition in response to sudden changes in CO_2 that have been reported in natural populations (Tortell and Morel 2002). When direct responses to fitness are measured in this experiment, sorting occurs primarily during the acclimation period and is not taken into account in the calculation of the direct response, which measures the improvement over the ancestor that is attributable to continued selection resulting in the appearance of novel types not seen in the founding population.

In this study, we investigated the effect of selective history on adaptive outcomes and found that all lines were able to adapt to low CO_2, and reached similar endpoints regardless of their history of selection. In addition, three of the four Low selection lines measured evolved higher-affinity net CO_2 uptake relative to their ancestor at low CO_2, which may partly explain the direct response to selection. The finding that both Low(High) and Low(Amb) lines show a direct response to selection at Low CO_2, and that a subset show an improvement in a system thought to be under selection demonstrates that long term decreases in CO_2 alone can produce evolutionary change in microalgae with CCMs. This suggests that microalgae in the recent past were different than contemporary algae. In addition, the Amb(High) lines returned to the same fitness as the Amb(Amb) lines, demonstrating that the High selection phenotypes were evolutionarily reversible in terms of fitness. Taken together, this demonstrates that over the range of CO_2 and time investigated, selective history did not appear to constrain the ability of *Chlamydomonas* to adapt to lower CO_2 environments, measured as growth in pure culture. It also demonstrates that fitness can be recovered quickly (about 175 generations) by natural selection in large populations. This is consistent with the findings of microevolutionary experiments, where fitness between replicate lines tends to converge when they are selected in the same environment (Travisano and Lenski 1996; Travisano 1997; Riley at al. 2001; Cuevas et al. 2002), and loss of fitness is usually reversible (Estes and Lynch 2003).

In this system, adaptation to a given level of CO_2 is specific to that level. Tradeoffs are a general feature of adaptation to specific levels of CO_2, which we have previously reported in High adapted lines (Collins and Bell 2004). In this case, some of the Low selection lines failed to grow reliably at levels of CO_2 higher than the one at which they were selected. A less severe tradeoff is

that a minority of Low selection lines did not show a detectable increase in growth rates with increasing CO_2. These results are in agreement with the evolution of specialist and generalist types in microevolutionary experiments (Reboud and Bell 1997), where constant environments lead to the evolution of specialists whereas environments that fluctuate in time permit the evolution of generalists. The rapid evolution of specialists for different levels of CO_2 is consistent with several levels of induction of a CCM over a range of CO_2 levels, rather than all or nothing CCM induction (reviewed in Matsuda et al. 1998). If the CCM was simply turned on at ambient levels of CO_2 and continued to be turned on at low CO_2, one would expect all Low selection lines to be able to grow at ambient CO_2 and vice versa. This points towards the existence of a subset of CCM genes that are specific to Ambient and/or Low CO_2, which is supported by some CCM knockout phenotypes. For example, the pmp-1 knockout grows at very low, but not at ambient, levels of CO_2 (Spalding et al. 2002). Tradeoffs associated with adaptation to a specific level of CO_2 suggest that a highly-regulated algal CCM may have evolved as a result of fluctuations of CO_2, which would be likely to occur in blooming phytoplankton, rather than in response to the mean concentration of CO_2 present in the environment. Studies on *Peridinium gatunense* showed an increase in CCM activity during an annual bloom in natural populations, although CO_2 limitation leading to oxidative stress eventually led to a population crash (Berman-Frank et al. 1995).

In addition to tradeoffs in growth at different CO_2 concentrations, we show that CCM regulation is affected by selection at Low CO_2. Of the lines investigated, all Low selection lines (regardless of ancestor) and one Amb(High) line gained CCM regulation. Higher-affinity CO_2 uptake was induced at ambient CO_2, but was not further improved at Low CO_2; this is qualitatively similar to

wild-type regulation we measured in *Chlamydomonas*. However, in the Low selection lines, this was done by decreasing the CO_2 uptake affinity at High CO_2 by at least 6.6-fold relative to their ancestor. This suggests that fitness recovery happened by compensatory mutations rather than by backmutation. Backmutation would have led to a wild-type phenotype with ancestral values of CO_2 uptake affinity at high CO_2 instead of loss of function in the ancestral environment. Compensatory mutations have been shown to be responsible for fitness recovery in experiments with viruses and microbes (Elena et al. 1998; Burch and Chao 1999; Moore et al. 2000; Rokyta et al. 2002) as well as metazoans (Estes and Lynch 2003). The presence both CO_2 sensitive and insensitive Low selection lines demonstrates that how *Chlamydomonas* responds to changes in CO_2, though predictable at physiological time scales, is fundamentally changeable over evolutionary ones.

In this experiment, both backselection and selection at low CO_2 resulted in evolved lines with a range in growth rates that were either sensitive or insensitive to CO_2 concentration. Along with differences in growth, selection at low CO_2 led to at least two different types of carbon uptake kinetics (CO_2 sensitive, and CO_2 insensitive). Thus, the variability seen in the affinity and regulation of CCMs in microalgae could be partially attributable to neutral variation produced by chance during repeated changes in CO_2 and bicarbonate levels. Examples of both CO_2 sensitive and insensitive growth have been reported in natural populations of phytoplankton (Hein and Sand-Jensen 1998; Tortell and Morel 2002), as have different CCM regulation strategies, even among phytoplankton with similar biology (Raven et al. 2002; Raven 2003; Rost et al. 2003). It is clear that historical factors constrain adaptive outcomes over very long time scales in phytoplankton, as can be seen by differences in Rubisco affinities between taxa, where affinity

correlates with atmospheric CO_2 at the epoch when each taxon emerged (Tortell 2000). However, our data suggest that the diversity of CO_2 growth and uptake strategies may not be attributable to selective history or adaptation over shorter time scales, but are instead attributable to chance events, even in closely related populations.

Our results show that history does not affect adaptive outcomes measured as fitness over microevolutionary time scales in this system. However, over a time scale of hundreds of generations, Amb(High) lines did not regain the CO_2 uptake characteristics usually seen in standard lab populations of *Chlamydomonas*. Like backselection experiments in *Drosophila* (Teotónio et al. 2002; Estes and Lynch 2003), this suggests that complex phenotypes are unlikely to be faithfully restored by backselection. We have demonstrated that chance events can drastically change the affinity and regulation of carbon uptake, as well as the CO_2 dependence of growth rates, after only about 1200 generations of selection. This diversity of phenotypes occurs without systematically affecting fitness, and corresponds qualitatively to growth and CO_2 uptake strategies seen between contemporary taxa of phytoplankton. More experimental work over a range of environments is needed to understand the role of reverse selection and chance in explaining the biology and diversity of contemporary populations.

Figure legends

Fig. 2 A: Maximum growth rate of Low(Amb) and Low(High) lines. B: Maximum sustainable cell densities in Low(Amb) and Low(High) lines. In both cases, each point represents a single selection line. Horizontal lines show mean across all lines ± s.e.m. Values are relative to Amb(Amb) lines growing at ambient CO_2. Each point represents a mean value from at least 30 replicate cultures in 2 independent assays.

Fig. 3: Direct response to selection at low CO_2 in Low(Amb) and Low(High) lines, shown either as change in maximum sustainable cell density or maximum growth rate. The direct response to selection is calculated from average absolute values of characters as follows: (derived-ancestor)/ancestor. Each point represents a single selection line. Horizontal lines show mean across all lines ± s.e.m.

Fig. 4: Response to backselection in Amb(High) lines, measured as either maximum growth rate or maximum sustainable cell population size at ambient CO_2. Control lines are Amb(Amb) lines, which were grown at ambient CO_2 for the duration of the selection experiment. In both cases, each point represents a single selection line. Horizontal lines show mean across all lines ± s.e.m. Values are relative to Amb(Amb) lines growing at ambient CO_2. Each point represents a single selection line mean value from at least 30 replicate cultures in 2 independent assays.

Fig. 5: $K_{0.5}$ of net CO_2 uptake. In all cases, solid bars represent ancestor or wild type populations, white bars immediately following represent lines derived from that ancestor. Bars represent mean ± s.e.m. from two or three independent replicates. A: Net CO_2 uptake affinity of

Low(Amb) and Low(High) lines at low CO_2. White bars represent lines selected at low CO_2. B: Net CO_2 uptake affinity at ambient CO_2 in Backselection lines. C: Net CO_2 uptake affinity of Amb(High), Low(Amb) and Low(High)Low, and ancestor lines at High CO_2. D: Maximum fold increase in net CO_2 uptake affinity ($K_{0.5}$) in wild-type, High (ancestral), Amb(Low), High(Low), and High(Amb) lines. Fold increase is calculated as ($K_{0.5}$ at high CO_2/$K_{0.5}$ at selected low CO_2). Each bar represents mean ± s.e.m. for all lines from a given selection regime.

Figure 1

Selection experiment

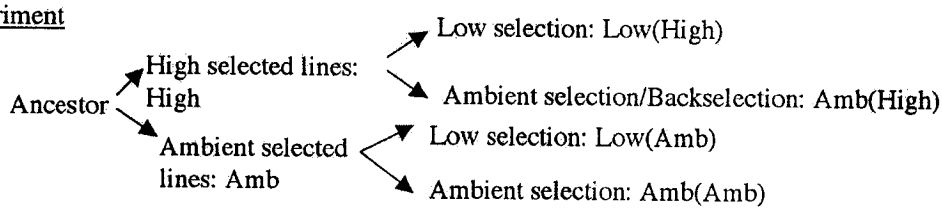
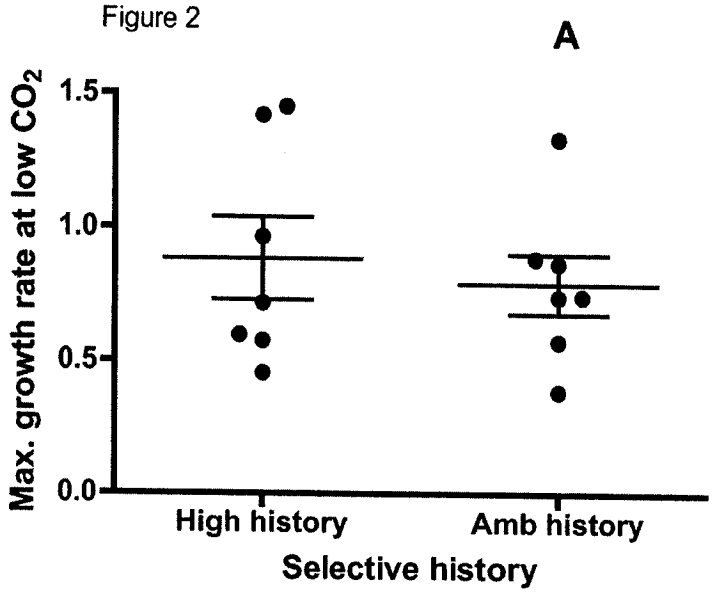


Figure 2


A
Max. growth rate at low CO_2
1.5
1.0
0.5
0.0
High history
Amb history
Selective history

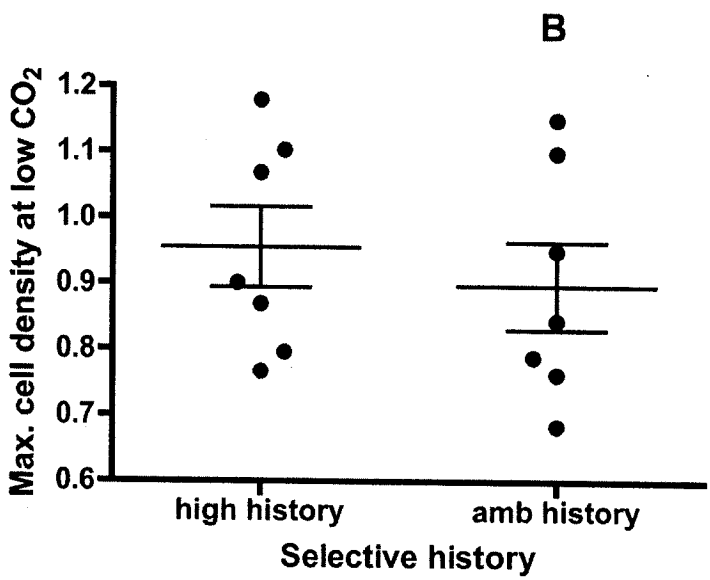

B
Max. cell density at low CO_2
1.2
1.1
1.0
0.9
0.8
0.7
0.6
high history
amb history
Selective history

Figure 3

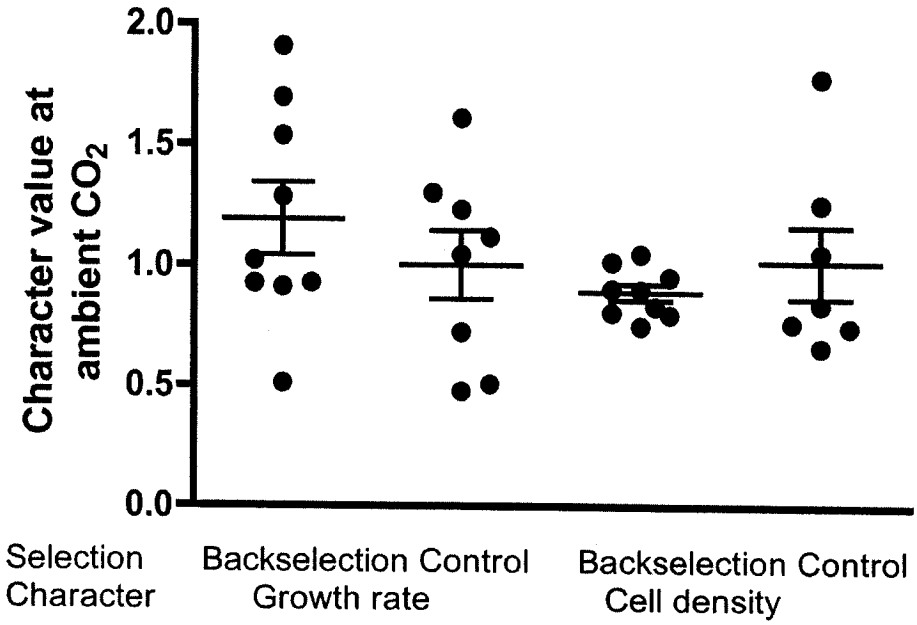

2.0
1.5
1.0
0.5
0.0
Character value at ambient CO_2
Selection Character
Backselection Control Growth rate
Backselection Control Cell density

Figure 4

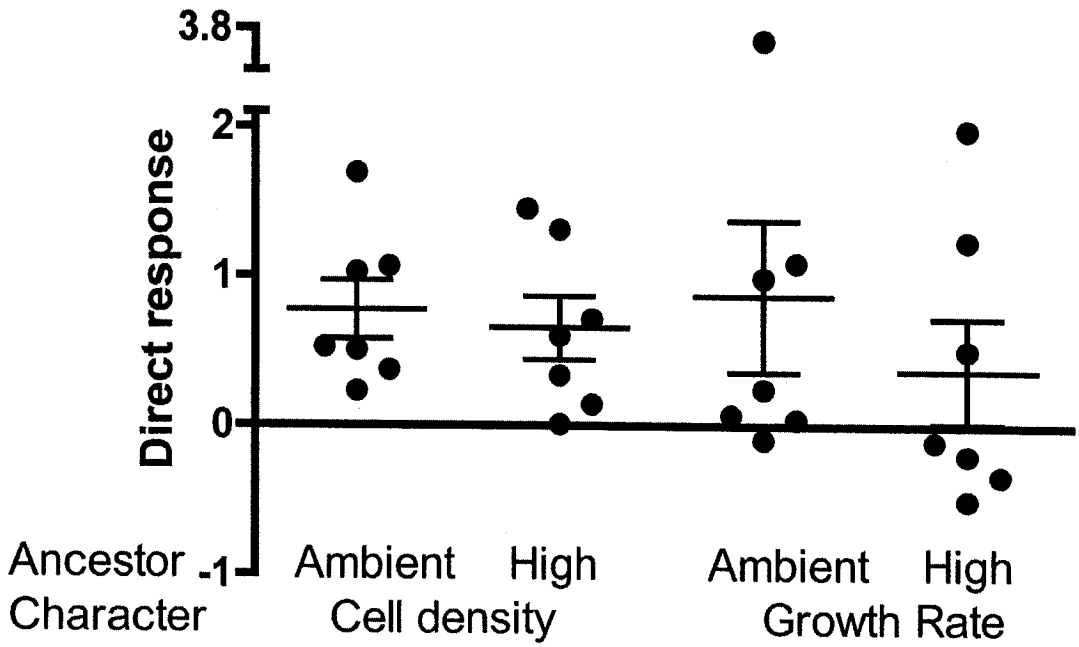

3.8
2
1
0
-1
Direct response
Ancestor Character
Ambient
High
Cell density
Ambient
High
Growth Rate

Figure 5

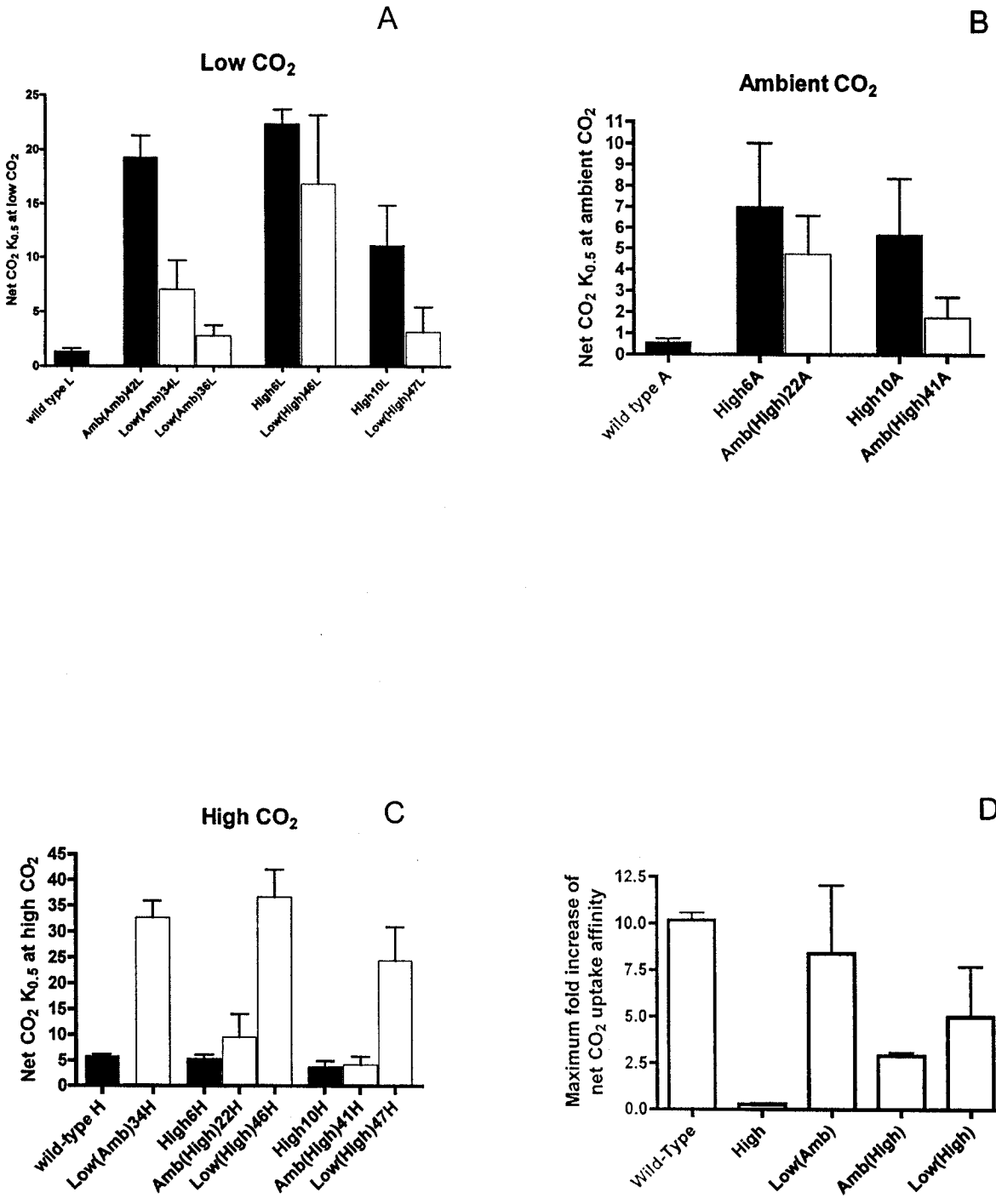

A
Low CO_2
Net CO_2 $K_{0.5}$ at low CO_2
wild type L
Amb(Amb)42L
Low(Amb)34L
Low(Amb)36L
High6L
Low(High)46L
High10L
Low(High)47L
B
Ambient CO_2
Net CO_2 $K_{0.5}$ at ambient CO_2
wild type A
High6A
Amb(High)22A
High10A
Amb(High)41A
C
High CO_2
Net CO_2 $K_{0.5}$ at high CO_2
wild-type H
Low(Amb)34H
High6H
Amb(High)22H
Low(High)46H
High10H
Amb(High)41H
Low(High)47H
D
Maximum fold increase of net CO_2 uptake affinity
Wild-Type
High
Low(Amb)
Amb(High)
Low(High)

Table 1: Estimated maximum frequencies of low CO_2-tolerant types in ancestors of Low CO_2 selection lines. Each frequency represents a mean value ± s.e.m. from two independent replicates.

Selection Line	Selected Environment	Estimated frequency of types able to grow at Low CO_2
4	high CO_2	>0.5
11	ambient CO_2	0.045 ± 0.022
12	ambient CO_2	0.012 ± 0.004
20	ambient CO_2	>0.5

Table 2: Summary of responses to selection. Genetic responses relative to ancestor are in bold. Physiological responses due to environment are in italics.

Selection(History)	Environment		
	Low	Ambient	High
Amb(Amb)	•*Reduced growth.*	•Control for Amb(High) backselection growth and regulation.	•Control for Amb(High). •*Increased growth*
Amb(High)	•*Reduced growth.*	**•Increased growth, higher affinity CO_2 uptake. Same phenotypes as Amb(Amb).**	**•Same growth and CO_2 uptake as Amb(Amb).** •*Increased growth*
Low(Amb)	**•Increased growth, higher affinity CO_2 uptake.**	**•Range of responses, including no growth.** •*Higher affinity CO_2 uptake*	**•Range of growth rates including 0 •Lower affinity CO_2 uptake.**
Low(High)	**•Increased growth, higher affinity CO_2 uptake.**	•Range of responses. •*Higher affinity CO_2 uptake*	**•Range of growth rates •Lower affinity CO_2 uptake.**

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Linking section 5

The work in much of this thesis was done because microalgal responses to CO_2 enrichment over the next century or two are likely to be ecologically important, and have the potential to affect large-scale processes such as carbon cycling and other nutrient cycles. As such, it was important to verify if the evolutionary responses obtained in the laboratory can also be found in natural populations that have also experienced long-term elevated CO_2. Chapter 5 describes the evolutionary response of natural populations of microalgae isolated from CO_2 springs.

Chapter 5: Evolution of natural algal populations at elevated CO_2

This chapter has been submitted to *Ecology Letters* as a manuscript by S. Collins and G. Bell.

Abstract

Over the next century, it is expected that the concentration of CO_2 in the atmosphere will roughly double (Watson et al., 2001). Microbial populations, which have large population sizes and short generation times, may respond to CO_2 enrichment through genetic change. Here we describe microalgae isolated from the soil of natural CO_2 springs and compare these strains with lines of *Chlamydomonas* that were selected at elevated CO_2 in the laboratory. Both the laboratory and natural populations failed to evolve specific adaptations to elevated CO_2, and contain populations that grow poorly at ambient levels of CO_2. Laboratory and CO_2 spring populations also include lines whose growth rates are insensitive to CO_2. This demonstrates that, although laboratory selection experiments use simplified environments, the evolutionary responses that are seen following long-term CO_2 enrichment correspond to those found in natural populations that have experienced similar conditions.

Introduction

Laboratory selection experiments are often used to understand better the processes that underlie adaptation. In contrast, selection experiments can also be used to obtain or describe a particular phenotype that is important for commercial, medical or ecological reasons. This is the case with experimental evolution studies examining the degradation of pollutants (Swenson et al. 2000) or antibiotic resistance (Chopra et al., 2003; Dobrindt et al., 2004) in bacteria, or responses to rising CO_2 in algae (Collins and Bell, 2004). In cases such as these, it is important to establish that the model system used mimics the outcome of selection in natural populations closely enough to produce realistic end outcomes.

Experimental approaches are powerful because they allow the experimenter to control the environment, and thus to attribute adaptive outcomes unambiguously to a defined environmental change. However, by defining and controlling the environment that adaptation occurs in, experimental systems run the risk of oversimplifying important aspects of the natural environment. This oversimplification may alter adaptive outcomes, which need not be a weakness in experiments meant to study a general process, but is of concern when specific adaptive outcomes are being studied. We previously used an experimental model system in order to study the evolutionary effects of elevated CO_2 on microalgae (Collins and Bell, 2004). The main simplifications used in the experimental design were that the algae populations were grown at a pH of 7 that did not change as CO_2 increased and were grown in a nutrient-rich media. Furthermore, they existed in a microcosm where no competition with other species could occur, although this has been shown to be important in multicellular plants as well as in mixed cultures of microalgae acclimating to changes CO_2 levels (Bazzaz, 1995; Tortell, 2002). The

conditions of our previous experiment were not intended to mirror the natural growth conditions of microalgae accurately, but to detect any evolutionary change attributable solely to elevated CO_2. A more fundamental limitation of experimental evolution as a whole is that it may provide a range of possible outcomes, but cannot predict which outcome(s) will occur in nature (with respect to plant responses to elevated CO_2 see for example Klus et al. 2001; Collins and Bell 2004)

CO_2 springs offer a naturally-occurring selection experiment, where populations have existed at elevated CO_2 for hundreds of years or more (Raschi et al. 1997), which is ample time for evolutionary change to occur in microorganisms or plants with short generation times. Material from CO_2 springs has been used in order to understand better long term genetic changes that occur in response to increased CO_2 (Raschi et al. 1999). This work has focused primarily on multicellular plants, although a few studies of photosynthetic algae found in lichen in CO_2 springs have been published (Balaguer et al. 1999; Balaguer and Barnes 1999). Responses observed in multicellular plants from CO_2 springs have been shown to be comparable to those observed in microevolutionary experiments spanning several generations (Marchi et al. 2004). One of the weaknesses in using CO_2 springs alone to look at evolutionary responses is that it is very difficult to attribute responses definitively to high CO_2 (see for example Andalo et al. 1999). Parameters that co-vary with CO_2 levels, such as pH or the presence of competing species can confound the cause of microevolutionary change and may also make it difficult to find an appropriate ambient CO_2 control population nearby. It is also difficult to be sure that heritable physiological differences translate into differences in fitness, rather than to neutral variation between populations (Woodward, 1999; Fordham and Barnes, 1999). In addition, unknown

migration rates from nearby populations adapted to ambient CO_2 levels may impede adaptation to high CO_2 in resident populations. Despite these ambiguities, CO_2 springs offer long-term mesocosm experiments that should be taken advantage of, both as experimental systems on their own and in combination with laboratory studies. Here, we have isolated soil microalgae from two well-studied CO_2 springs in order to characterize their growth and compare it with laboratory selection lines.

In contrast to studies using multicellular plants, almost all work to date on microalgal responses to rising CO_2 has been done using short-term studies in natural and lab populations (reviewed recently by Beardall et al., 1998; Colman et al. 2002; Riebesell, 2005), or by comparisons between different taxa of extant phytoplankton (Raven 2003). These studies have resulted in detailed descriptions of the physiological responses of contemporary populations to changes in CO_2. The strength of this approach is that it has established that the changes in carbon uptake in standard microalgal model systems behave like natural populations over short timescales. In this study, we show that the same holds true for using the model alga *Chlamydomonas* to experimentally test long-term evolutionary responses to increasing CO_2 in microalgae. In demonstrating that the model system we have used to date behaves like comparable natural populations, we show that CO_2 springs and laboratory experiments can be used together in order to obtain both a realistic description of a high CO_2 evolved phenotype, as well as explain how natural selection can lead to this phenotype.

Materials and Methods

Collection and isolation of natural populations. Soil samples were collected at the Bossoleto CO_2 spring near Sienna, Italy (Lat. 43°17', Long.11°35') and at the Strmec spring near Radenci, Slovenia (Lat.46°39',Long.16°00'). The Bossoleto and Strmec springs are well-characterized CO_2 springs with undetectable levels of methane and sulphur in their emissions. Average daytime CO_2 levels at the springs range from 600ppm to 1200ppm in air at the Bossoleto spring and 2600ppm to 4200ppm in soil at the Strmec spring (Bettarini et al., 1999; Kaligaric 2001; Marchi et al., 2004), though CO_2 levels in soil very near the springs can exceed 5%. Samples were collected in CO_2-enriched areas around the springs, and control cultures were collected in nearby areas that were not enriched. Soil samples were mixed with 1/4 strength HSM (Harris, 1989) and illuminated under either very high (5%) or ambient levels of CO_2 and sampled after 48 and 72 hours of growth. This level of CO_2 was within the range of CO_2 levels measured at the Bossoleto spring during sampling. Microalgae were isolated from soil by phototaxis (Sack et al., 1994). Algae were allowed up to 24 hours to swim though agarose-filled pipettes; cells that reached the illuminated end of the pipettes were then grown on HSM agar plates at either very high or ambient CO_2. This procedure would have failed to isolate any types that had an absolute (short-term) requirement for high CO_2 or which could not grow in HSM. Populations were identified by visual inspection by Thomas Pröschold (texts used: Ettl and Gärtner, 1988; 1995; Gärtner and Ettl, 1998). Isolates were nearly monospecific, made up of one of the following: *Tetracystis sp.*, *Chloronomala sp.* or *Chlorococcum sp.*, all of which are common soil algae (Bold and Wynne, 1985). Individual isolate identifications are listed in Appendix 1.

Maximum population densities and growth rates. Maximum population densities and growth rates were measured in 384-well plates containing 90μL HSM per well. All cultures were first acclimated for a single culture cycle (3-6 days), then diluted and transferred to assay plates. For high CO_2 evolved lines that often failed to grow at ambient CO_2, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays. This may have allowed some backselection of the high CO_2 evolved lines, so that the results presented here are conservative. The plates were grown in the same phytotron chamber as above at either 430ppm or 2000ppm CO_2. Absorbance of each culture was measured every 24 hours. Maximum densities were calculated from the maximum absorbance attained by a culture. Growth rates and maximum densities are relative, such that the average value of the control cultures growing at ambient CO_2 is 1.0. Three independent triplicate measurements were made for each culture.

Results

Growth rates and maximum population densities.

Figure 1 shows the growth rates and maximum population densities of CO_2 spring and control isolates. Both control and CO_2 spring populations were successfully isolated from Strmec soil samples, but no control populations were isolated from the Bossoleto spring. As such, only results are from the Strmec isolates are analyzed below.

There was no detectable effect of genus on either growth rates ($F_{5,5}$=1.83, p=0.19) or maximum population densities ($F_{5,5}$=1.24, p=0.35). Because of this, the isolates are only divided into CO_2 spring and control populations below, regardless of identity. This may be partially because the

isolation procedure used would have excluded cells that grew very slowly or were not motile, and any populations that could not grow detectably in the media used. Thus, the isolation procedure itself might select for relatively homogenous growth rates.

Figure 1a shows the relative growth rates of the spring and control isolates. The expected physiological response to an increase in CO_2 is an increase in growth rate. The effect of the assay environment (high or ambient CO_2) is significant ($F_{1,1}=17.95$, $p < 0.0001$) whereas the main effect of habitat (spring vs. control) is ambiguous ($F_{1,1}=3.48$, $p=0.068$) and the assay x habitat interaction is non-significant. A direct evolutionary response to long-term growth at high CO_2 would result in the CO_2 spring isolates having a higher growth rate or maximum population density than the control cultures at high CO_2. However, populations from the CO_2 springs on average have growth rate 31% lower than the control populations under high CO_2 conditions, though the range of growth rates does not extend below that of the controls. This effect is only marginally significant (see above), mostly because there is no systematic difference in growth rates between high and control isolates at ambient CO_2. There is no significant difference in maximum population densities achieved by the CO_2 spring isolates and the controls when grown at high CO_2.

Figure 1b shows the maximum cell density attainable by a culture. There is no significant difference in mean growth rates ($F_{1,1}=0.07$, $p=0.79$) or maximum population densities ($F_{1,1}=1.15$, $p=0.29$) between the CO_2 spring and control populations when grown at ambient CO_2. However, three out of ten CO_2 spring populations have near-zero growth rates at ambient CO_2, and

periodically fail to grow at all at ambient CO_2. These three isolates do not have unusually low growth rates when grown at high CO_2.

Changes in sensitivity to CO_2. Figure 2 shows the physiological response to changes in CO_2 in populations collected from either CO_2 springs or from surrounding areas where CO_2 was not elevated (control). The response is shown as either change in growth rate (Figure 2a) or change in maximum population size (Figure 2b). The physiological response is calculated as proportional increase in growth rate or maximum population size at high CO_2, such that a value of 0 indicates that growth rate or maximum population size is the same at high and ambient CO_2.

Both control and CO_2 spring populations were successfully isolated from Strmec soil samples. The following results are from the Strmec isolates only. There is no significant effect of genus on the physiological response to increases in CO_2 (Growth rate: $F_{5,5}=2.29$, $p=0.12$; Maximum population density: $F_{5,5}=1.05$, $p=0.44$), Because of this, the isolates are pooled into either spring or control treatments below, regardless of identity. We found no effect of long-term growth at high CO_2 on the mean physiological response to CO_2 levels in terms of growth rate ($t_{27}=1.3$, $p=0.21$) or maximum population densities ($t_{27}=0.67$, $p=0.51$). The populations isolated from the CO_2 spring did, however have a larger variance in the response of growth rate to CO_2 than did the control cultures ($F=6.64$, $p=0.016$), since the sample from the CO_2 spring contained both isolates with very reduced growth at ambient CO_2 (values at or near 1) and CO_2-insensitive isolates (values at or near 0), which the control sample did not.

As before, the Bossoleto samples are not analyzed quantitatively. Qualitatively, we see the presence of a high-CO_2-requiring isolate of *Chlorella minutissima* (value of 1), which does not grow at all at ambient CO_2, though standard lab populations of *Chlorella* are usually cultured in air. *Chlorella sp.* was not isolated in any of the control samples from either spring.

Discussion

In this study, natural isolates from populations representing three genera of microalgae did not show any evidence of adaptive evolutionary change that could be attributed to elevated CO_2. This is in good agreement with the results obtained from a laboratory selection experiment carried out at elevated CO_2 over 1000 generations in *Chlamydomonas*, a widely-used model system for photosynthesis and carbon uptake (Collins and Bell, 2004). The phenotypes of the laboratory lines and natural isolates are summarized in Table 1. Neither laboratory selection lines nor CO_2 spring isolates show a direct response to selection at high CO_2 in terms of growth rate or maximum population density. Selection at high CO_2 resulted in an apparently maladaptive response in some cases, seen as significantly lowered population density in the laboratory cultures and as lowered growth rates in the CO_2 spring isolates. Both laboratory and CO_2 spring isolates had populations that failed to grow reliably at ambient CO_2, present at a frequency of 2/10 in the laboratory lines and 3/10 in the natural isolates from the Strmec spring, as well as one isolate from the Bossoleto spring.

Both laboratory and natural populations showed an apparently maladaptive response when grown at high CO_2: a reduction in mean maximum population density in the laboratory populations and a reduction in mean growth rate in the natural isolates. This suggests that some

loss of function in carbon uptake or metabolism is a general feature of long-term exposure to elevated CO_2 in microalgal populations. It has been suggested that contemporary populations of plants are adapted to lower (preindustrial or Pleistocene) CO_2 levels, and that this may constrain or preclude adaptive evolution to elevated CO_2 (Sage and Coleman 2001). While this study does not address the reason that both natural and lab populations of microalgae failed to adapt to elevated CO_2, it does empirically show that adaptation is unlikely to occur over thousands of generations. This is in agreement with studies spanning a comparable number of generations which were carried out using banked seeds from the mid 19th century (Balaguer and Barnes 1999), and in multicellular plants found at CO_2 springs (Augusti et al., 1999; Andalo et al., 1999; Belaguer and Barnes, 1999), though failure to adapt to elevated CO_2 is not always the case (Tousignant and Potvin, 1996; Polle et al., 2001;Vodnik et al., 2002).

Though there is no adaptive response to growth at elevated CO_2, a range of phenotypes can be seen in populations propagated at high CO_2, such as isolates that are unable to grow at ambient CO_2. All of the control isolates are able to increase their growth rate in response to an increase in CO_2, whereas two of the CO_2 spring isolates are unable to do so. These growth-CO_2 relationships do not systematically correspond to growth rate or maximum population density at high CO_2. A similar range evolved among replicate experimental lines of *Chlamydomonas* (Collins and Bell, 2004), demonstrating that long-term responses to elevated CO_2 vary among natural populations in the same way that they do between experimental lines in the laboratory.

Because there is a range of neutral phenotypes, long-term growth at elevated CO_2 has the potential to cause non-adaptive evolutionary change and phenotypic divergence in natural

populations of microalgae as well as in a laboratory model system. The repercussions of this could be especially important in isolated populations of the same species, which have the potential to diverge as CO_2 levels rise. The range of neutral phenotypes found in high CO_2 populations introduces a source of uncertainty and the possibility of multiple simultaneous outcomes into predictions of how microalgal populations are likely to change as CO_2 rises. This uncertainty should not be mistaken for a lack of understanding, or be used as an excuse either to discount models based on current growth-CO_2 relationships, or to fail to take prudent measures that could manage anthropogenic impacts on natural systems. However, findings such as the ones in this study highlight the inherent unpredictability of biological systems, even when they have been greatly simplified for laboratory experiments.

In the natural isolates described here, microalgal populations are unable to improve their performance significantly at elevated CO_2, but this does not exclude the possibility that elevated CO_2 could indirectly cause adaptive change. A negative correlated response, where some CO_2 spring isolates show reduced growth at ambient CO_2, shows that some degree of normal function can be lost when CO_2 is constantly abundant. Similarly, it was found that *Chlamydomonas* selected at high CO_2 lost the ability to induce high-affinity CO_2 uptake as effectively as wild-type cells (Collins, Sültemeyer and Bell, unpublished). This might allow energy diverted from CO_2 uptake or regulation to be devoted to the acquisition of limiting resources, such as iron (Falkowski, 1994; Riebesell, 2004). This hypothesis has yet to be directly tested in microalgae, though there is some support for it in land plants grown in competition under high CO_2 for several generations (Bazzaz et al. 1995).

The goal of this study was to compare the evolutionary responses of natural and laboratory populations of microalgae to elevated CO_2. For the laboratory selection experiment, a common algal model system, *Chlamydomonas reinhardtii*, was used. *Chlamydomonas* is a common soil alga that possesses the ability to actively concentrate inorganic carbon inside its cell (Badger et al., 1980). Two of the three genera of soil algae isolated from the Strmec spring, *Chlorococcus* and *Tetracystis*, are part of the Order Chlamydomonales along with *Chlamydomonas* (Lewis and McCourt, 2004). *Chlorococcus sp.* is known to induce active concentration of carbon inside its cell under low CO_2 (Miyachi et al. 2003), as does *Chlamydomonas*. To the best of our knowledge, the carbon uptake physiology of *Tetracystis* or *Chloronomala* has not been reported, although the ability to concentrate inorganic carbon is present in most microalgae studied to date (Colman et al., 2002). In multicellular plants, responses to CO_2 measured over several generations vary idiosyncratically between species (reviewed by Ward and Strain, 1999; Urban, 2003). In contrast with this, our current study failed to detect any systematic between-genus differences in evolutionary responses to elevated CO_2. This suggests that the range of results obtained using replicate laboratory populations approximates the range of results found in the same size sample of natural populations with similar biology.

Experimental studies designed to examine the effects of specific environmental changes must simplify both the biotic and physical environment in order to draw clear conclusions about the nature and cause of adaptation. While this simplification is necessary, it is important to establish to what extent model systems behave like the natural populations that they are meant to represent. Our findings validate the use of a model system for studying the evolutionary response

of algae to elevated CO_2 and verifies that the phenotypes found in the laboratory correspond with the outcome of selection in natural communities.

Figure legends

Fig 1 A: Relative growth rates of CO_2 spring and control algae at high (2000 ppm) and ambient levels of CO_2. B: Relative maximum cell density attainted by cultures of CO_2 spring and control algae at high and ambient levels of CO_2. In both cases, each point represents the mean value from three independent replicate measurements for a single population.

Fig 2 A: Response of growth rate to an increase in CO_2 concentration from ambient to high (2000 ppm) in CO_2 spring and control cultures. B: Response of maximum cell density to an increase in CO_2 concentration from ambient to high in CO_2 spring and control cultures. In all cases, a value of 0 denotes no change between ambient and high CO_2, a value of 1 denotes no growth at ambient CO_2. Each point represents the mean value from three independent replicate measurements for a single population.

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Figure 1

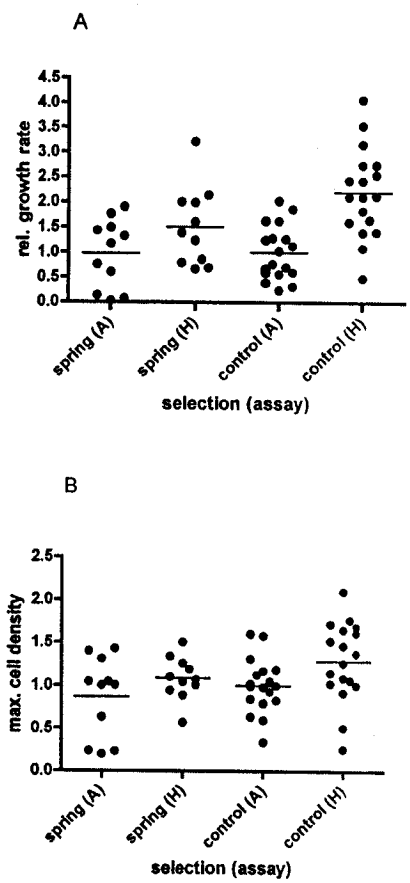

Figure 2

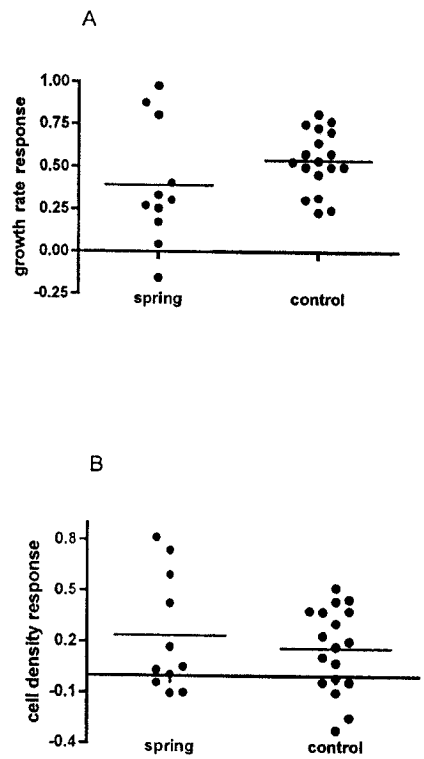

Table 1

	Characters at high CO_2	Characters at ambient CO_2	Response to changes in CO_2
Laboratory selection	• No adaptive response to selection • Lower average limiting density • Higher variance in limiting density	• Presence of high CO_2-requiring lines	• Presence of CO_2 insensitive lines
CO_2 spring	• No adaptive response to selection • Lower average growth rate • No difference in variances	• Presence of high CO_2-requiring lines	• Presence of CO_2 insensitive lines

Appendix 1: Identification of soil microalgae

identification	reference
Apodochloris polymorpha (Bischoff et Bold) Komarek 1979	Ettl & Gärtner (1988, 1995)
Chlorella minutissima Fott et Novakova 1969	Ettl & Gärtner (1995)
Chlorococcum echinozygotum Starr 1955	Ettl & Gärtner (1988, 1995), Gärtner & Ettl (1988)
Chlorococcum infusionum (Schrank) Meneghini 1842	Ettl & Gärtner (1988, 1995), Gärtner & Ettl (1988)
Chlorococcum minutum Starr 1955	Ettl & Gärtner (1988, 1995), Gärtner & Ettl (1988)
Chlorococcum novae-angliae Archibald et Bold 1970	Ettl & Gärtner (1988, 1995), Gärtner & Ettl (1988)
Chlorococcum pinguideum Arce et Bold 1958	Ettl & Gärtner (1988, 1995), Gärtner & Ettl (1988)
Chloronomala cupreola Groover et Bold 1969	Ettl & Gärtner (1995)
Chloronomala palmelloides Mitra 1950	Ettl & Gärtner (1995)
Friedmannia israeliensis Chantanachat et Bold 1962	Ettl & Gärtner (1995)
Pseudochlorococcum polymorphum Archibald 1970	Ettl & Gärtner (1995)
Tetracystis aggregata Brown et Bold 1964	Ettl & Gärtner (1988, 1995)
Tetracystis excentrica Brown et Bold 1964	Ettl & Gärtner (1988, 1995)
Tetracystis fissurata Nakano 1984	Ettl & Gärtner (1988, 1995)
Tetracystis illinoisensis Brown et Bold 1964	Ettl & Gärtner (1988, 1995)
Tetracystis pampae Brown et Bold 1964	Ettl & Gärtner (1988, 1995)

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General Conclusion and Summary

Phytoplankton responses to elevated CO_2 is a topic that has received considerable attention in ecology, plant physiology and oceanography over the past decade. This is partly because responses to changes in CO_2 shed light on the current biology and ecology of phytoplankton. However, the main reason for the attention that "high CO_2" studies receive is because of the rapid rate of CO_2 increase that is being experienced at a global level, and the potential for oceans to act as carbon sinks that may help manage global CO_2 enrichment. Despite this, there are surprisingly few studies that span long enough periods of time to directly test how phytoplankton may evolve in response to elevated CO_2.

The responses to long-term growth at elevated CO_2 in unicellular algae follow a general pattern. There is no adaptive response to growth at elevated CO_2, but there is a range of non-adaptive phenotypes. These phenotypes differ from those seen following short-term acclimation to elevated CO_2, and fall into two qualitative categories: one category can be described as CO_2 insensitive, and show no differences in growth rates or photosynthetic rates when grown at ambient and elevated levels of CO_2; the second category can be described as inefficient, and shows increased rates of photosynthesis without an increase in growth when grown at elevated CO_2. On average, lines selected at elevated CO_2 have smaller population sizes. These phenotypes are attributable, at least in part, to the inability to induce high affinity CO_2 uptake. This loss of regulation is not reversed by selection at lower levels of CO_2, though constitutive high-affinity CO_2 uptake can be restored after a few hundred generations of growth at lower CO_2.

Since there is no adaptive response to elevated CO_2, an increase in CO_2 is thought to relax stabilizing selection that maintains the regulated carbon uptake seen in contemporary populations. This presumably allows for the accumulation of conditionally neutral mutations in genes that affect CO_2 uptake and metabolism. In addition, the lack of an adaptive response to elevated CO_2 allows replicate populations to diverge, with the differences between replicate populations being attributable mainly to chance, rather than to historical factors. Though experimental evolution studies such as this one can present a range of possible phenotypes, they cannot predict which will occur in natural populations.

One of the main difficulties in integrating evolutionary responses into the existing literature on phytoplankton responses to elevated CO_2 is that there is little explicit treatment on how or if short-term processes can be scaled up to provide meaningful predictions of long-term responses. The results presented in this thesis suggest that adaptive responses differ unpredictably in both sign and magnitude from acclimation responses, and that scaling up from short-term studies may not be realistic. At least from a theoretical standpoint, it also seems that the rate of environmental change used in experiments may itself affect adaptive outcomes, and that this should be taken into account in the design of future competition and selection experiments.

My main conclusions from this work can be summarized as follows:

1. Microalgae do not show an adaptive response to elevated CO_2. This is supported by results from laboratory selection lines and natural populations isolated from high CO_2 springs.

2. Non-adaptive responses resulting from long-term growth at elevated CO_2 differ in magnitude, and sometimes sign, from acclimation responses described in the physiology literature.
3. Long-term growth at elevated CO_2 results in decreased fitness in the ancestral environment, which appears to be attributable to the accumulation of conditionally neutral mutations.
4. The decrease in fitness in the ancestral (ambient CO_2) environment can be at least partially explained by the loss of the ability to induce high-affinity CO_2 uptake and/or by cells that leak CO_2. Both of these characters describe some degradation of the carbon concentrating mechanism (CCM), which is found in almost all phytoplankton studied to date.
5. The decrease in fitness and loss of high-affinity CO_2 uptake seen after long-term growth at elevated CO_2 are reversible, though the loss of CCM regulation is not. However, lines can adapt to ambient or even to Pleistocene levels of CO_2 by constitutively increasing the affinity of CO_2 uptake.
6. Chance, rather than selective history, explains divergence between replicate lines that are adapting to changes in CO_2.